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REFLEX STIMULATION AND INHIBITION OF RETICULUM CONTRACTIONS IN THE RUMINANT STOMACH

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Contractions of the reticulum, one of the anterior divisions of the ruminant stomach, generally occur in conscious animals at regular intervals of 30–120 sec, according to the conditions at the time of observation. They are known to be abolished by bilateral cervical vagotomy (Mangold & Klein, 1927; Duncan, 1953) and after the administration of atropine.

Within the past few years there have been a number of reports that contractions of the reticulum occur in decerebrated or anaesthetized animals and that they may be evoked in these preparations by stimulation, in an afferent sense, of vagal fibres in the abdomen or neck (Iggo, 1951, 1956; Titchen, 1953; Dussardier & Albe-Fessard, 1954). Alterations in the conditions within the different divisions of the stomach have been shown to excite or inhibit such contractions in decerebrate or anaesthetized preparations (Titchen, 1953, 1954; Dussardier, 1955).

In this paper an account is given of experiments in which a variety of stimuli applied to different parts of the ruminant stomach resulted in the reflex excitation or inhibition of contractions of the reticulum. Preliminary accounts of some of these experiments have been presented previously (Titchen, 1953, 1954).

METHODS

The animals used were young and adult goats, sheep and calves 2–10 weeks old. The younger animals were fed on milk and hay, the older animals on grass or hay supplemented with a protein-rich pellet diet. Food but not water was withheld in the 24–48 hr before an experiment.

Anaesthesia was induced with ethyl chloride and maintained with ether. Decerebrate preparations were made as previously described (Comline & Titchen, 1951*a*).

Contractions of the reticulum were recorded from a water manometer connected by an air-filled tube to a balloon containing water. This balloon was inserted into the reticulum through an incision about 1·5 cm long in the region of its free pole. It was kept in position with a purse-string suture tied around the polythene tube to which the balloon was attached. The capacity of the balloons used varied from 100 to 700 ml. at 10–20 cm water pressure. In some experiments, and particularly those in which the responses of the reticulum to stretch of itself were studied, care had to be taken to avoid the overflow of fluid from the manometer during contractions of the reticulum. For this reason the simple closed system of recording from a balloon containing water and connected by an air-filled tube to a water manometer was modified to include an open reservoir. This modification is shown in Fig. 1, and has been referred to as the open system of recording. The presence of this reservoir in the recording system protected the water manometer during contractions of the reticulum when large volumes of water were displaced from the balloon. Records obtained with this open system gave a less accurate representation of the form and force of the contractions of the reticulum than those obtained with the simpler closed system. With both systems the records obtained did not measure exactly the actual form and force of the contractions of the reticulum but were probably more accurate than those which would have been obtained from balloons inflated with air. The records were obtained with pressures of 5–15 cm water in the balloons; at these pressures slight relaxations of the reticulum, which could be observed, were sometimes recorded.

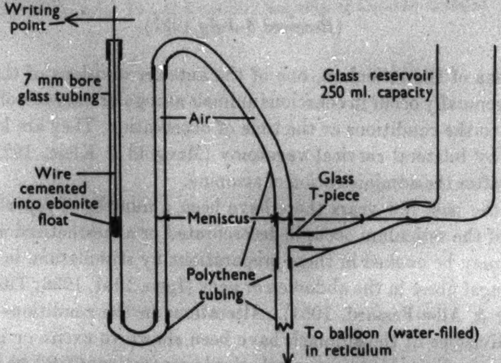
Writing point
7 mm bore glass tubing
Wire cemented into ebonite float
Air
Meniscus
Polythene tubing
Glass reservoir 250 ml. capacity
Glass T-piece
To balloon (water-filled) in reticulum

Fig. 1. The reservoir-manometer system sometimes used to record the reflex contractions of the reticulum, and referred to, in the text, as the open system of recording.

Nerve stimulation was carried out in the manner described previously (Comline & Titchen, 1951*a*). L-Adrenaline base (British Drug Houses) and the sympatholytic substance phentolamine (Rogitine, Ciba) were dissolved with HCl in 0·9 % NaCl solution and diluted to an appropriate concentration with saline before injection through a cannula into a femoral vein. Atropine sulphate (British Drug Houses) was dissolved in saline. Blood pressure was recorded with a mercury manometer connected to a cannula inserted into a femoral or carotid artery.

When required the abomasum was isolated by ligatures tied about the pylorus and at the omaso-abomasal junction. Care was taken to avoid the inclusion of major vessels and nerves in these ligatures. Samples of the contents of the abomasum were obtained through a cannula tied into a relatively avascular part of its ventral surface. The abomasum was washed out at the beginning of an experiment with successive volumes of 100–500 ml. of 0·9 % NaCl at 38° C. 25–100 ml. of saline at 38° C was introduced into the abomasum and withdrawn after 10–30 min in experiments in which the secretory activity of the abomasum was followed. These samples were titrated against 0·05 N-NaOH using the Topfer-phenolphthalein indicator as described by King (1951) to titrate free and total acidity of the washings. The progressive diminution in the strength of acid introduced into the abomasum was determined by titration against N-NaOH. pH was determined electrometrically.

RESULTS

Contractions of the reticulum which involved the whole of the organ, similar to those recorded in conscious animals, occurred in decerebrate preparations of goats and sheep, and also of calves over 2 weeks of age. The contractions usually began within 10–30 min of the laparotomy to expose the reticulum and occurred at regular intervals of 20–180 sec. When first recorded they were usually diphasic contractions of the musculature of the entire organ. Such contractions, observed in conscious animals, have been referred to in the literature as total contractions (Mangold & Klein, 1927; Phillipson, 1939). These contractions were present in many, but not all, experiments for 30–180 min after decerebration under ether anaesthesia. After this time they ceased. The contractions diminished in force or frequency, or both, and often changed from their original diphasic to a monophasic form before they ceased. Such a series of contractions was observed in four preparations in which respiration ceased after decerebration and did not return until after a period of 60–120 min of artificial ventilation. In two decerebrate preparations, one of a calf and one of a goat, the reticulum contractions continued largely undiminished in force and frequency for 300 min after decerebration. The activity of the reticulum was not recorded and the operative procedures undertaken were only those necessary to carry out the decerebration and to observe, but not to record, from the reticulum in these two experiments. In calves the two phases of the diphasic contractions were separated by a complete relaxation, but in sheep and goats there was only a partial relaxation between them. Monophasic contractions were usually complete within 3–5 sec of the start of the contraction; relaxation and contraction occupied about equal times. Diphasic contractions occurred over a period of 7–10 sec. The first and second phases each occupied about 3–5 sec.

Direct observation of the reticulum revealed that there were increasingly intense contractions of localized regions of the reticulum before the onset of a total contraction. Localized activity of the reticulum was sometimes recorded as a slight increase in the base line of the manometer tracing for a period of 5–25 sec before the occurrence of the total contraction. The localized contractions of the reticulum were markedly reduced after each total contraction.

The effect of afferent stimulation from the gut was first studied on the series of contractions which occurred after decerebration. The contractions were inhibited by manipulation of the abdominal viscera, by laparotomy incisions and by distension of the abomasum. They were increased in force and frequency after the addition of fluid to the balloon in the reticulum, after moderate stretch of the omasum, the introduction of a tube into the oesophagus or the introduction of acid into the abomasum. When the contractions had ceased they reappeared with the delivery of one of these stimuli.

The decline in the force and frequency of the contractions of the reticulum observed in a number of experiments in the period immediately after decerebration was accompanied by a decline in the amount of acid detected in successive samples from the abomasum. The progressive decline in the force and frequency of reticulum contractions after decerebration and the diminution in the amounts of acid in successive samples from the abomasum in one experiment is shown in Fig. 2. Both the reticulum contractions and acid secretion from the abomasum waned over a period of 30–180 min after decerebration. An examination of the free and total acid content of successive samples obtained from the abomasum revealed that the total amount and duration of acid secretion by the abomasum was smaller in sheep than it was

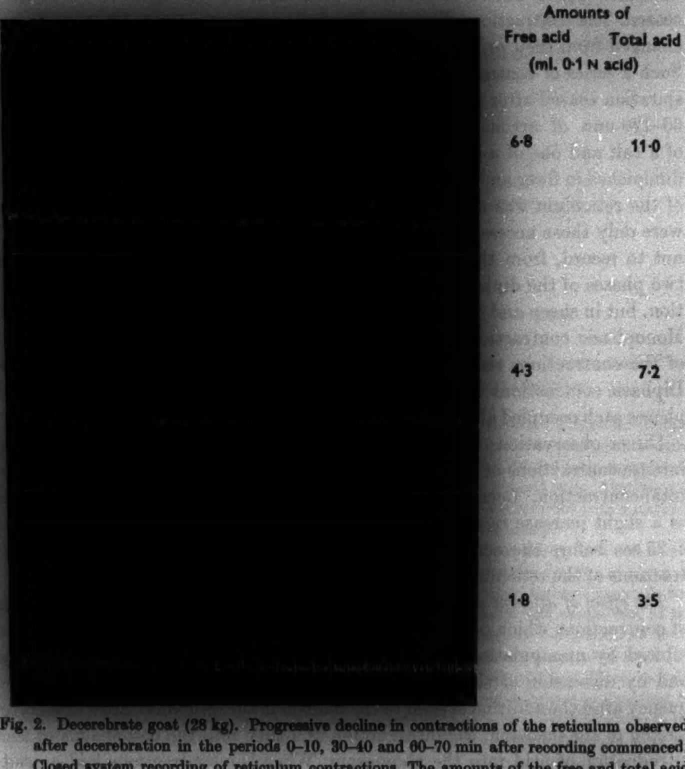

Fig. 2. Decerebrate goat (28 kg). Progressive decline in contractions of the reticulum observed after decerebration in the periods 0–10, 30–40 and 60–70 min after recording commenced. Closed system recording of reticulum contractions. The amounts of the free and total acid detected in the abomasum in these periods are shown to the right of the tracings.

in goats after decerebration; the force and frequency of the reticulum contractions declined more rapidly in sheep after decerebration than it did in goats. These observations led to an examination of the effects of the introduction of acid into the abomasum. The conditions under which this proved a stimulus to reticulum contractions are described below.

Reflex stimulation of reticulum contractions

Vagal stimulation. Contractions of the reticulum could be evoked, or when already present, increased in force or frequency, by stimulation of the central end of an abomasal or a reticulo-omasal branch of the ventral abdominal vagus nerve, or by stimulation of the central end of the ventral abdominal vagus nerve itself (Fig. 3). The reaction to stimulation of these nerve trunks varied. Frequently a short period of stimulation produced a number of contractions of the reticulum, although sometimes only one contraction followed. With either prolonged or repeated short periods of stimulation a series of contractions could be evoked. These contractions rarely occurred at intervals of less than 30 sec and sometimes continued after the stimulation ceased.

Fig. 3 Decerebrate goat. Effect of stimulation of central end of the main abomasal branch of the ventral abdominal vagus nerve (signal), neon tube stimulator 40/sec. Records from above down: closed system record of reticulum contractions, signal, time marker 30 sec.

The first contraction which occurred in response to stimulation of one of these afferent nerves was often monophasic; later contractions were diphasic. There was a variable delay between the application of the stimulus and the response. It was usually greatest when nerve stimulation was first undertaken in an experiment and least after a series of contractions had been evoked or when the effects of afferent nerve stimulation were studied during a pre-existing, or previously stimulated, series of contractions. The latent period thus varied from 2 to 20 sec. The response to afferent nerve stimulation also varied according to the frequency of the stimulation. Excitatory effects were obtained with frequencies of stimulation of 20–60/sec, but with higher frequencies and particularly those above 120/sec there was either an inhibition or no response to stimulation of, for example, the abomasal nerve.

The responses of the reticulum to stimulation of these nerves were abolished by the administration of atropine (0·2–0·4 mg/kg). Reflex contractions of the reticulum were not observed in preparations in which the dorsal abdominal vagus nerve had been cut and only the ventral abdominal vagus nerve left intact. Stimulation of the peripheral end of the ventral vagus nerve elicited slight contractions of the reticulum. Both the response to stimulation of the peripheral end of the ventral abdominal vagus and the greater contraction of the reticulum evoked by stimulation of the peripheral end of the dorsal abdominal vagus nerve were abolished by the administration of atropine (0·2–0·4 mg/kg).

Acid in the abomasum. The introduction of HCl or H_2SO_4 into the abomasum elicited contractions of the reticulum when the volume and strength of acid introduced were sufficient to lower the pH of the contents of the abomasum to 0·9–1·0 or less. Thus the acids were not effective in the presence of large volumes of abomasal contents, nor when introduced when phosphate buffer was already present in the abomasum. Their effect was terminated by the introduction of saline or of a buffer solution into the abomasum.

The responses of the reticulum to acid introduced into the abomasum usually occurred within 30–75 sec, rarely up to 180 sec, when the first of a series of contractions of the reticulum was observed. This contraction was usually smaller than those which followed in the next 3–4 min, after which time the contractions declined in force or frequency or both, as the strength of the acid in the abomasum decreased. They usually ceased when the pH of the abomasal contents rose to about 1·1–1·2 (Fig. 4). Reticulum contractions were not stimulated by the introduction of acid into the abomasum after the abomasal branches of the ventral abdominal vagus nerve had been cut. In these experiments the abomasum had been isolated from the rest of the stomach and from the duodenum by ligatures tied around the pylorus and at the omaso-abomasal junction.

Contractions of the reticulum sometimes occurred after the abomasal mucosa had been touched with the end of a glass rod or a polythene tube. The nature of the stimulus was not defined but the response was associated with the movement of the rod or tube rather than with pressure. In a number of experiments movement of the abomasal cannula evoked contractions of the reticulum.

Stretching the reticulum. The first contraction of the reticulum observed in an experiment frequently occurred when the organ was stretched as it was lifted in order to introduce a balloon into it. The possibility that stretching the reticulum would stimulate its reflex contractions was therefore examined in further detail.

A series of contractions of the reticulum followed an increase in the volume of fluid in the reticulum balloon. If contractions of the reticulum had already ceased they were increased in frequency when this procedure was adopted. The effect was usually apparent within 30–90 sec of the addition of the fluid to the balloon. The contractions usually reached a maximum force and frequency within 5–6 min of the application of the stimulus and continued for 30–50 min, and in some experiments in which the balloon was distended to a greater degree for up to 150 min, in both cases gradually waning in frequency and sometimes also in force over this period (Fig. 5). In a number of preparations there was an increase in the force of the contractions as they decreased in frequency.

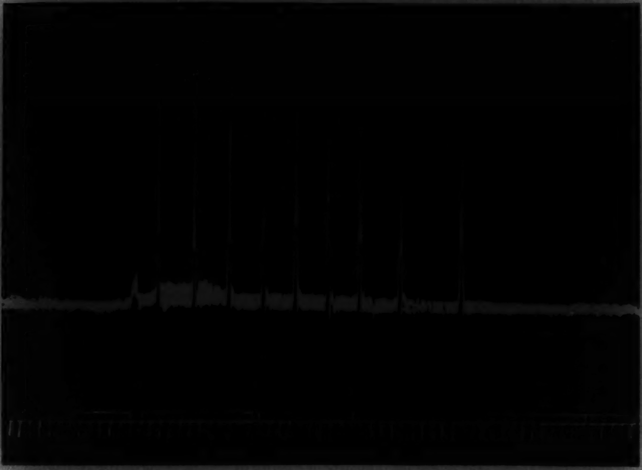

Fig. 4. Decerebrate sheep. Reflex contractions of the reticulum stimulated by the introduction of HCl into the abomasum. Open system recording of reticulum contractions, time marker 30 sec. At the point marked *a*, 50 ml. 0·2 N-HCl was introduced into the abomasum; contraction of the reticulum occurred 60 sec later. The pH of the samples of the contents of the abomasum withdrawn at the points marked were *b*, 0·87; *c*, 1·1; *d*, 1·4.

The stimulus appeared to be associated with the change in the volume in the balloon. Thus if the balloon were returned to its original volume during a series of contractions, these ceased (Fig. 6). Again, if the fluid was withdrawn from the balloon after the reflex response had ceased, the inflation 20–60 min later with the same volume would again evoke a series of reflex contractions, if the balloon had in the meantime remained empty or had contained a considerably reduced volume of fluid.

Responses of the reticulum to stretch were obtained in preparations in which the ventral abdominal vagus nerves had been cut. The contractions of the reticulum observed were less forceful and sometimes also less frequent than those stimulated by the same degree of stretch before this nerve was cut. After the ventral abdominal vagus had been cut a greater degree of stretch was required to evoke reticulum contractions. They were not observed at all in those preparations in which the dorsal abdominal vagus nerve had been divided. The administration of atropine (0·2–0·4 mg/kg) resulted in the cessation of the reticulum contractions stimulated by stretch.

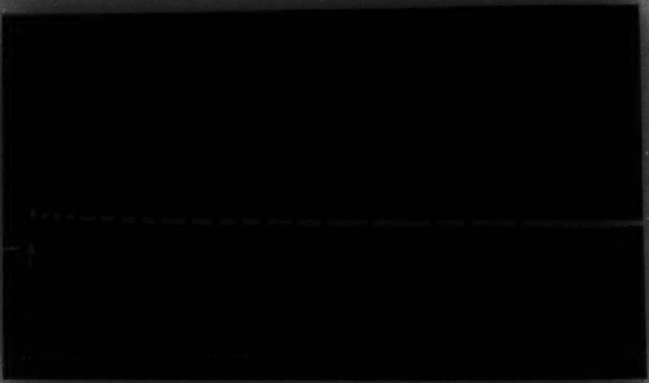

Fig. 5. Decerebrate sheep (35 kg). Records as in Fig. 3. The reflex response of the reticulum to stretching. At the point marked by the arrow the volume of water in the reticulum balloon was increased from 200 to 400 ml. The first of a series of contractions of the reticulum occurred 35 sec later. The last contraction shown in this record occurred 39 min after the volume of the reticulum balloon was increased.

Stimulation of the omasum. In three experiments the inflation of a balloon introduced into the omasal canal was followed by an increase in the force and frequency of reticulum contractions. In decerebrate preparations squeezing the omasum frequently stimulated swallowing movements which were sometimes accompanied by a mono- or di-phasic contraction of the reticulum. The stimulus was difficult to repeat many times in one experiment, as the omasum tended to retain its deformed shape and then further pressure applied to it was ineffective. The responses to this form of stimulation of the omasum were not obtained after section of the ventral abdominal vagus nerve.

Stimulation of the oesophagus. When a tube was introduced through the mouth into the terminal part of the thoracic oesophagus, up to five or six contractions of the reticulum occurred. The response was associated with the movement of the tube in this terminal part of the oesophagus. Contractions were not elicited if the tube remained stationary or was moved in another region of the oesophagus; moderate distension of a balloon introduced into the oesophagus was ineffective. The contractions which occurred in response to this stimulus frequently showed three or more phases and were also usually more forceful than the diphasic contractions elicited with the other stimuli described.

Fig. 6. Decerebrate goat. Open system record of reticulum contractions. Effect of a short period of distension of the reticulum balloon. The volume of water in the balloon was increased from 50 to 100 ml. at the first arrow and returned to 50 ml. 8 min later at the second arrow. Time marker 30 sec.

Inhibition of reticulum contractions

The insertion of cannulae into the abomasum, and other procedures which involved manipulation of the abdominal viscera, particularly of the abomasum or the pylorus, produced a profound inhibition of reticulum contractions which frequently persisted for 20 min or more. It was largely avoided by carrying out such procedures either before or immediately after decerebration whilst the ether anaesthesia persisted.

Inhibition due to distension of the abomasum could also be shown by inflation of a balloon in it or by the introduction of 200–1000 ml. 0·9% NaCl at 38° C, according to the size of the animal. The effect was apparent within 30–60 sec and persisted throughout periods of distension of up to 20 min. In some experiments the reticulum contractions were completely abolished by the distension. This was the case with greater degrees of distension; a lesser volume in the abomasum caused a reduction of the force or frequency or both of reticulum contractions (Fig. 7).

In two experiments in which the abomasum was divided across its longitudinal axis into two approximately equal parts, with as little interruption of its blood and nerve supply as possible, the introduction of acid into either half of the abomasum elicited contractions of the reticulum, which could be inhibited by distension of the other part.

Fig. 7. Decerebrate sheep (17 kg). Reticulum contractions stimulated by stretch (500 ml. water introduced into balloon at *R*), inhibited completely by distension of abomasum (signal) with 500 ml. saline at 38° C (*A* and *B*), and partially inhibited by distension of the abomasum with 250 ml. saline (*C*). Closed system of recording of reticulum contractions, time marker 60 sec.

Section of the splanchnic nerves affected the responses to distension of the abomasum. In three goats and two sheep, in which the splanchnic nerves had been cut at operations 2 weeks to 3 months previously, distension of the abomasum produced a predominantly excitatory response and no longer caused a complete inhibition of the contractions of the reticulum. An increase in the frequency of the contractions of the reticulum was observed in all five preparations on distension of the abomasum; in two they were slightly reduced in force and in the rest increased in force (Fig. 8). Similar results were obtained in three preparations in which the splanchnic nerves were cut during the course of the experiment.

The effect of stimulation of the central end of a splanchnic nerve was examined in three experiments. The start of a period of stimulation was marked by a pronounced contraction of the abdominal muscles and succeeded by a period of inhibition of the contractions of the reticulum (Fig. 9). This result of stimulation of the central end of a splanchnic nerve was obtained in two preparations in which the other splanchnic nerve had also been cut.

In four decerebrate preparations, two of goats, two of sheep, the effect of distension of the abomasum was altered by section of the major abomasal branch of the ventral abdominal vagus nerve. After this nerve had been cut the inhibitory effects of abomasal distension were less marked, and the complete inhibition was replaced by a slight reduction in the force with sometimes a slight increase in the frequency of the reticulum contractions.

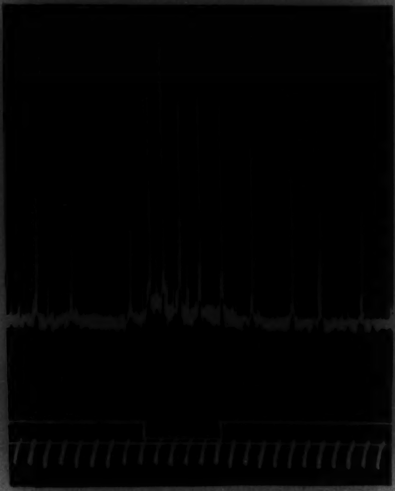

Fig. 8. Decerebrate goat (21 kg); both splanchnic nerves had been cut at an operation two weeks before the experiment. Effect of distension of abomasum with 300 ml. saline at 38° C (signal). The stimulus to reticulum contractions before distension of the abomasum was stretch (200 ml.). Closed system recording of reticulum contractions, time marker 30 sec.

The increase in frequency of reticulum contractions with abomasal distension was not observed in preparations in which the whole ventral abdominal vagus nerve trunk had been cut in the first part of its course in the abdomen immediately beyond the oesophageal hiatus of the diaphragm.

In experiments in which the effects of abomasal distension were examined the abomasum had been isolated between ligatures tied at the pylorus and at the omaso-abomasal junction. It proved important to ensure that these ligatures had been effective. In two cases the ligatures failed to prevent the escape of fluid from the abomasum. In these experiments, after the abomasum was emptied there was a period during which the reticulum contractions occurred at a markedly increased frequency. All the fluid introduced to distend the abomasum was not recovered from it; in both experiments some escaped into the omasum and in one into the duodenum also. The ligature tied at the omaso-abomasal junction was found at the end of one of these experiments to have displaced the fold of abomasum which appears to serve usually as a flap valve to prevent the regurgitation of abomasal contents into the omasum.

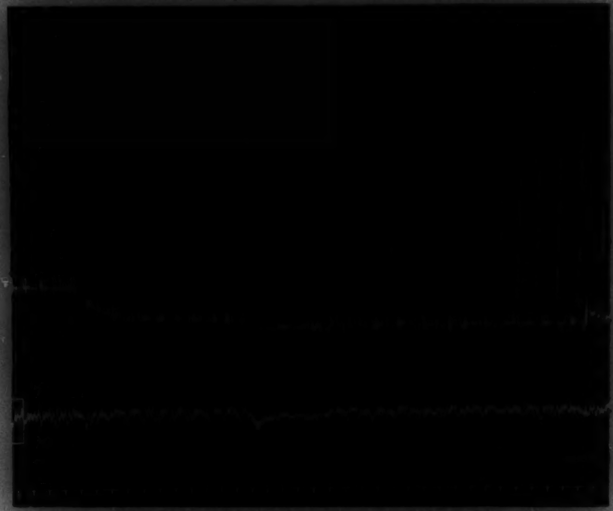

Fig. 9. Decerebrate sheep (17 kg). Response of reticulum to stretch (400 ml. into balloon at *A*) showing effect of stimulation of central end of left splanchnic nerve for 5 min, signal (induction coil, 11 cm). Elevation in reticulum record marked by arrow was due to a contraction of abdominal muscles. Records from above down: closed system record of reticulum contractions, carotid arterial blood pressure, signal, time marker 60 sec.

Only slight changes in the blood pressure followed distension of the abomasum. These were rarely as great as 10 mm Hg and usually consisted of an initial slight fall, succeeded either by a continued slight depression, or by a return to the previous or a slightly increased level. Stimulation of the central end of a splanchnic nerve was followed by a sustained slight fall in the blood pressure with, initially, a slowing of the heart (Fig. 9).

In addition to the immediate effects of a period of distension of the abomasum, changes in the reticulum contractions after its relief were observed. These after-effects were apparent for 3–5 min or more after the withdrawal of fluid from the abomasum. After one or two less forceful contractions of the reticulum, there was an increase in the force with a decrease in the frequency of the reticulum contractions (Fig. 7*B*), an increase in both force and frequency of reticulum contractions (Fig. 7*A*), or in their frequency (Fig. 10*A*) or in their force. The excitatory effects of abomasal distension in preparations in which the splanchnic nerves had been cut also continued after the abomasum was emptied (Fig. 8). In contrast there was a persistent reduction in both the force and frequency of reticulum contractions after their complete inhibition during stimulation of a central end of a splanchnic nerve (Fig. 9). The persistent after-effects of these stimuli were not accompanied by any similarly prolonged changes in blood pressure.

Fig. 10. Decerebrate sheep (15 kg). Response of reticulum to stretch (250 ml.) interrupted by two periods of abomasal distension, each with 250 ml. saline at 38° C (signal) before (*A*) and after (*B*) the major abomasal branch of the ventral abdominal vagus nerve was cut at *a*. Closed system recording of reticulum contractions, time marker 30 sec.

Pyloric inhibition. The most profound and persistent form of inhibition encountered was that which arose from manipulation, or dissection about the region, of the pylorus. In a number of those experiments in which a ligature had been tied about the pylorus there was either no response to stimuli, such as acid introduced into the abomasum or stretching the reticulum, or the number and force of the reflex contractions was very much smaller than was usually the case. The magnitude and duration of the responses were markedly increased after all the visible nerves to the region of the pylorus had been cut and the pylorus had been separated from its mesenteric attachments, or after the infiltration of 1% procaine beneath the serous surface of the pylorus. The pyloric inhibition was reduced by section of both splanchnic nerves or of the ventral abdominal vagus nerve. It was encountered in two preparations in which both splanchnic nerves and the ventral abdominal vagus nerve had been cut. The differences in the responses of the reticulum to the introduction of acid into the abomasum before and after denervation of the pylorus are shown in Fig. 11.

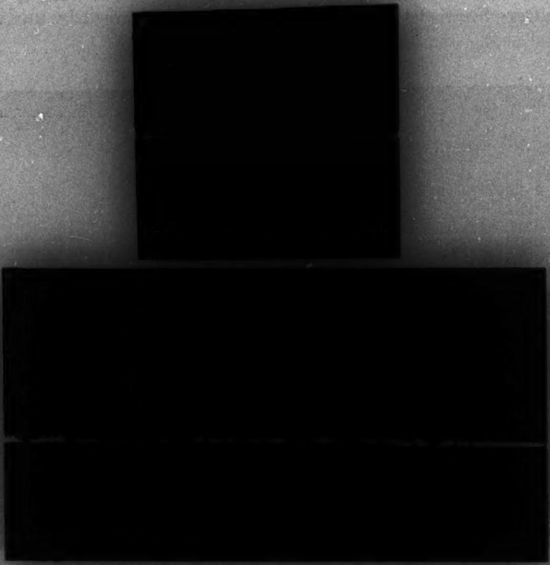

Fig. 11. Decerebrate sheep. Pyloric inhibition. A ligature had been tied about the pylorus. The two parts of the record show the response to the introduction of 50 ml. of 0·2 N-HCl into the abomasum before (*A*) and after (*B*) denervation of the pylorus. The pH of the first sample of the abomasal contents withdrawn after the introduction of acid into the abomasum (indicated by *a* on the record) was in the first instance 0·83 and in the second 0·82. After denervation of the pylorus the duration and magnitude of the responses of the reticulum were increased. Open system of recording from the reticulum, time marker 30 sec.

The effect of stimulation of the peripheral end of the splanchnic nerves or of the injection of adrenaline on the contractions of the reticulum. Contractions of the reticulum were completely or partially inhibited by either the intravenous injection of adrenaline or stimulation of the peripheral end of the splanchnic nerves. After the injection of 2–10 μg/kg of adrenaline the inhibition persisted for from 5 to 10 min. That produced by stimulation of the splanchnic nerve was reduced and shortened by removal of the adrenal gland of that side.

The inhibition from both sources was usually preceded by a slow contraction of the reticulum which resembled those produced by similar stimulation in spinal preparations (Comline & Titchen, 1951*b*) and can be seen in Fig. 12. Such a slow contraction was also observed in some decerebrate preparations in which contractions of the reticulum had not occurred for some time. It occurred at about the same time as the development of the pressor response (Fig. 13).

Fig. 12. Decerebrate goat (12 kg). Effect of adrenaline (2 μg/kg) injected intravenously (signal, Adr) on a series of reticulum contractions stimulated by stretch (250 ml.). Records from above downwards: closed system record of reticulum, femoral arterial blood pressure, time marker 10 sec.

In addition to these inhibitory effects, the intravenous injection of adrenaline was, in some preparations, followed by a series of contractions of the reticulum. This was most frequently observed in preparations in which the reticulum had failed to respond to stimuli which were normally effective in the production of reflex contractions. For example in one such experiment there was no response of the reticulum to stretch of itself within 3 min and an intravenous injection of 2 μg/kg of adrenaline was then made. During the pressor response a contraction of the reticulum occurred which was succeeded by a series of contractions. These responses were accompanied by a slight secretion of acid by the abomasum in two experiments. In one of these before the injection of adrenaline no acid had been detected in saline introduced into, and later removed from, the abomasum. In the saline left in the abomasum for 17 min after the adrenaline was injected the titration figure for total acid was 1·5 ml. 0·1 N acid, in the next 29 min 2·6 ml., and in the next 30 min 1·4 ml. 0·1N acid. These figures correspond to the periods between the signal marks Adr and A, A and B, B and C in Fig. 14. Such an excitatory effect of adrenaline was usually observed in preparations in which a profound inhibition was apparently present. It was never seen in preparations in which the vagus nerves had been cut or atropine administered.

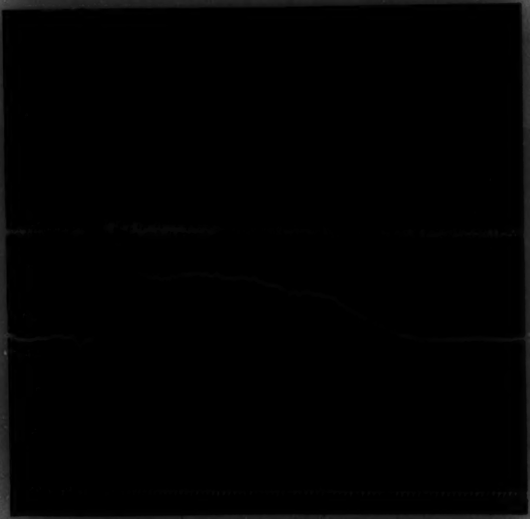

Fig. 13. Decerebrate goat (18 kg). Adrenaline contraction of the reticulum. Signal marks the injection of 5 μg/kg of adrenaline intravenously. Open system recording, femoral arterial blood pressure, time marker 10 sec.

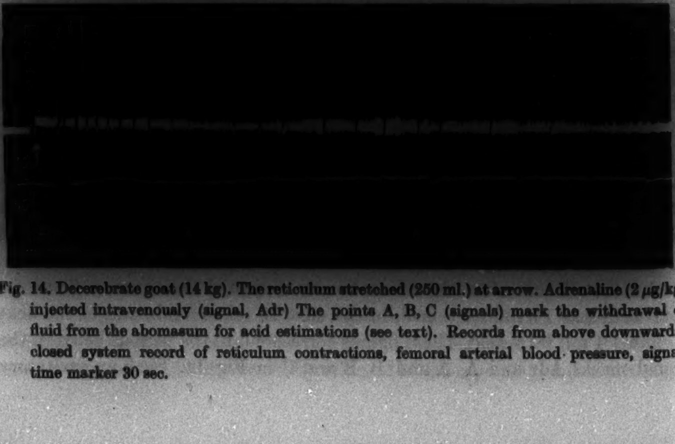

Fig. 14. Decerebrate goat (14 kg). The reticulum stretched (250 ml.) at arrow. Adrenaline (2 μg/kg) injected intravenously (signal, Adr) The points A, B, C (signals) mark the withdrawal of fluid from the abomasum for acid estimations (see text). Records from above downwards: closed system record of reticulum contractions, femoral arterial blood pressure, signal, time marker 30 sec.

DISCUSSION

The evidence obtained in the present experiments indicates that the characteristic diphasic contractions of the reticulum observed in decerebrate preparations are reflex contractions and that they result from the summed effects of afferent stimulation, both excitatory and inhibitory, from different parts of the stomach. This conclusion is based, in part, on the results of stimulation and section of branches of the vagus nerves. Stimulation of the ventral vagus nerve in an afferent sense elicited reflex reticulum contractions mediated by efferent cholinergic parasympathetic fibres distributed to the reticulum in the dorsal vagus nerve. Such experiments demonstrate the reflex stimulation of reticulum contractions, but do not necessarily establish the course of all the afferent and efferent nerve fibres which may be involved in the reflex responses. Afferent and efferent vagal fibres of the reticulum are distributed in both the dorsal and ventral abdominal vagus nerves.

The efferent fibres appear to be confined to the vagus nerves. Reflex contractions could not be elicited after both the dorsal and ventral abdominal vagus nerves had been cut. No evidence was obtained that such contractions depended on the integrity of the adrenergic sympathetic innervation of the reticulum. These results are in agreement with those obtained in unanaesthetized animals in which reticulum contractions were absent after the vagus nerves had been cut (Mangold & Klein, 1927; Duncan, 1953), but still present after the splanchnic nerves had been cut (Duncan, 1953). They also indicate that the contractions stimulated were reflex responses and not local responses of the organ, of either a myogenic or neurogenic origin.

The reflex centre or centres are probably situated in the medulla oblongata; they are less likely to be in the pons which suffers a variable degree of damage during decerebration by mechanical transection. The contractions described were absent in the spinal preparations used in a study of the efferent innervation of the reticulum (Comline & Titchen, 1951*b*). They were present in decerebrate preparations from which the cerebellum has been removed (personal observations) and in decerebrate preparations in which the cervical spinal cord has been transected (Iggo, 1951; personal observations). This conclusion on the site of the reflex centre is supported by the evidence obtained by Bell & Lawn (1955) who recorded contractions of the oesophagus, reticulum and rumen following stimulation through electrodes inserted into the medulla oblongata.

The stimuli which excite reflex reticulum contractions via vagal afferent fibres include stretching the reticulum, touch applied to the abomasum or to the oesophagus apparently close to the site of the sphincter described by Dougherty & Meredith (1955) and a reduction in the pH of the abomasum to between 0·9 and 1·0. The pH which is effective in the stimulation of reticulum contractions is lower than that of the abomasal contents of the conscious, fed animal; Masson & Phillipson (1952) found this to be 2·3–2·4. It is, however, close to that of 0·9 advanced by Hollander (1949) and Conway (1953) for the pH of the secretion of the oxyntic cells in a number of species and also to the figure of 1·05–1·32 reported by Masson & Phillipson (1952) for the pH of the secretion obtained from a Hollander pouch prepared in the abomasum of a sheep. The discrepancy between the pH of the abomasal contents and that effective as a stimulus in these experiments might be explained if the receptors sensitive to this acid stimulation were close to the site of its secretion by the oxyntic cells. If this were the case then the acid secretion and not the pH of the abomasal contents would appear likely to be the effective stimulus in the conscious animal. Direct and unequivocal evidence for the existence of acid or other receptors in the stomach has not been provided. The present experiments and those in which the activity in vagal afferent fibres was recorded during changes within the stomach (Paintal, 1953, 1954; Iggo, 1954, 1956) provide indirect but strong evidence for their existence.

Striking features of the reflex responses of the reticulum are their enduring nature with certain stimuli, their long latencies and the prolonged course of an inhibition once established. No satisfactory explanation can be offered for these temporal sequences, but the prolonged effects of stretching the reticulum, for example, may be due partly to the properties of the receptors involved. Paintal (1953) showed that the stretch receptors of the cat's stomach adapt slowly with constant maintained stimulation. In contrast the responses of the reticulum to apparently tactile stimulation were difficult to maintain or to repeat with continued or interrupted stimulation. These observations may be an indication that visceral tactile and stretch receptors resemble in their reactions the rapidly adapting tactile receptors of the skin (Adrian, 1928) and the more slowly adapting stretch receptors in skeletal muscle (Matthews, 1931) respectively.

The inhibition of reticulum contractions by distension of the abomasum resembles that reported by Phillipson (1939) in the conscious sheep. The present experiments show that the majority of the afferent nerve fibres concerned in this inhibition are contained in the splanchnic nerves, and that in some experiments their removal did not simply lessen or remove the inhibition but revealed an excitation on distension of the abomasum. Thus there appear to be two effects of abomasal distension, an inhibition mediated principally by splanchnic and also by some vagal afferent fibres and an excitation mediated by vagal afferents. Further analysis of this effect is complicated by the difficulty of separating the effects of afferent and efferent fibres in the splanchnic nerves. The possibility cannot be dismissed that abomasal distension inhibited reticulum contractions by a reflex excitation of the sympathetic innervation of the reticulum brought about by splanchnic afferents. The available evidence does not suggest that this is the most probable explanation. Alternatively, the inhibition might be ascribed to effects on the reflex centre also mediated by afferent fibres in the splanchnic nerves. Some evidence in favour of this might have been obtained from the experiments in which stimulation of the central end of one splanchnic nerve inhibited reticulum contractions in preparations in which both had been cut. However, afferent fibres other than those from the abomasum would be stimulated under these circumstances; these observations did not provide a precise definition of the nature of the inhibition.

The profound inhibition which follows manipulation of the pylorus resembles in many respects that arising from distension of the abomasum and is probably similar in its nature. Section of the splanchnic nerves did not completely remove the inhibition, and the course of all the afferent nerve fibres responsible was not determined. The pyloric inhibition may effectively limit the demonstration of reflex contractions of the reticulum in a decerebrate preparation.

In the experiments reported here both excitatory and inhibitory effects of adrenaline on the reticulum were found. In many experiments its administration reduced or abolished reflex contractions; this was to be expected in view of previous reports of its action in the conscious animal (Magee, 1932). Two forms of excitatory response to adrenaline were also observed. One was the comparatively slow single contraction which was described in spinal preparations (Comline & Titchen, 1951*b*) and appears to be a direct response to adrenaline. The other effect was that of a single intravenous injection of adrenaline being followed by a series of diphasic contractions, which were dependent on the vagal innervation. This last effect might be attributed to circulatory changes either in the central nervous system or in the reticulum. Facilitation by adrenaline of the receptors which must be presumed to be present in the stomach must also be considered as a probable explanation of the effect. It is supported by the observations made by Paintal (1954) that adrenaline may facilitate the stretch receptors of the cat's stomach and by those of Kay (1955) on the parotid secretion of sheep in which the anatomical separation of the afferent and efferent nerves has allowed a more precise definition of such a facilitation of visceral receptors.

The reflex effects on the reticulum were obtained in preparations in which previous to the delivery of the stimulus the reticulum had been inactive. The contractions of the reticulum which were observed immediately after decerebration without the application of any definite stimulus resembled the reflex contractions in all respects, and it is possible that their decline in force and frequency and their cessation may be attributed simply to a simultaneous decline in acid secretion by the abomasum which occurred after decerebration. An extension of this argument to ascribe reticulum contractions in the conscious animal purely to reflex excitation requires, however, further experimental evidence.

SUMMARY

1. Reflex reticulum contractions have been evoked in decerebrate preparations of sheep, goats and calves by electrical stimulation of vagal afferent nerve fibres and by alterations in conditions in the stomach.

2. The contractions ceased after the administration of atropine and were absent in preparations in which the dorsal abdominal vagus nerve had been cut.

3. The contractions were stimulated by:

Stretching the reticulum, or distension of a balloon in the omasal canal;
A reduction in the pH in the abomasum to 0·9–1·0;
Touching the lower part of the thoracic oesophagus or the abomasal mucosal surface; or
Stretching the abomasum after section of the splanchnic nerves.

4. The reflex contractions were inhibited by distension of the abomasum when the splanchnic nerves remained intact, by manipulation of the pylorus and by stimulation of the central end of a splanchnic nerve.

5. Both excitatory and inhibitory effects of adrenaline on the reticulum contractions were observed.

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FACTORS AFFECTING HISTAMINE-STIMULATED GASTRIC SECRETION IN ANAESTHETIZED DOGS

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During a study of gastric secretion in anaesthetized dogs, each with a pyloro-duodenal ligature, intact vagi, and a stomach fistula, it was observed that the gastric secretory response to histamine could be profoundly influenced by the pre-operative dietary regime of the animals, the integrity of the vagal nerve supply and the location of the pyloric ligature.

METHODS

Diet before experiment. All dogs, on admission to the animal house, were put on a standard diet of 1 pint of milk in the morning followed by a single meal of meat in the evening, with water available at all times. The dogs were divided into two groups: group 1, 'starved' dogs—these animals were not given any food on the day before an experiment but water was available; group 2, 'fed' dogs—these animals were not starved the day before an experiment. In addition to the normal daily diet of milk and meat, an extra pint of milk was given in the evening about 12 hr before an experiment.

Operative procedure. The dogs were anaesthetized with chloralose 2% (w/v) and urethane 20% (w/v) 2 ml./kg given intravenously. The right femoral vein was cannulated and connected to a Palmer's Slow Injection apparatus. A constant intravenous infusion of histamine was given by this route. An efficient airway was maintained by putting a McGill's no. 10 cuffed endotracheal tube into the trachea. In some experiments both vagus nerves were exposed in the neck and prepared with loops of thread for subsequent section. The abdomen was opened with a left subcostal muscle-splitting incision and the pyloro–duodenal junction was gently occluded with a ligature of white cotton tape. A glass cannula was tied into the body of the stomach, near the greater curvature. In almost all dogs, including the 'fed' dogs, the stomach was found to be completely empty; in one or two dogs, debris, such as masses of straw or chips of wood, was found. The abdominal wound was closed around the cannula and the animal was then suspended on a frame to allow free drainage through the cannula. The stomach was washed out with warm isotonic saline given by a stomach tube passed down the oesophagus. The head was kept very low to allow free external drainage of saliva and pharyngeal mucus. Lastly a thermometer was put into the rectum and the animal kept warm with radiant heat. After the preparation just described the animal was left undisturbed for approximately an hour before the histamine infusion was begun. During this period fluid from the cannula was collected and tested for the presence of free acid. Histamine acid phosphate was then given at the rate of 5 μg/kg/min, calculated in terms of the base, and made up in sterile solution.

Once the histamine infusion was begun the gastric juice was collected every 15 min and its volume measured in a graduated cylinder. The concentration of free acid in the juice, expressed in m-equiv/l., was estimated by titration with 0·05 N-NaOH, using Töpfer's reagent as indicator, and the output of free acid was expressed as m-equiv/15 min. The figures in the tables are averages of two consecutive collection periods each of 15 min.

RESULTS

Histamine-stimulated acid gastric secretion in 'starved' dogs

The results of ten experiments using 'starved' dogs are shown in Table 1. Between the end of the operative procedures and the start of the histamine infusion no acid gastric juice was secreted by any of the dogs. In all experiments acid gastric secretion began within 0·5 hr of starting the infusion of histamine and the output of acid increased rapidly for 1·5 hr. Between 1·5 and 2 hr after the start of the histamine infusion, the output of acid rose more slowly in the majority of the experiments: in Expt. 2 a plateau was reached at 1·5 hr, and in Expt. 10 a slight fall in secretion occurred at 2·5 hr. Between 2·5 and 3 hr a fall in secretion was seen in most experiments. This fall was never more than 22 % of the previous maximum output. Thus the output of histamine-stimulated gastric acid in 'starved' anaesthetized dogs with intact vagi approximates to a steady state between 1·5 and 3·5 hr after the start of the histamine infusion.

TABLE 1. Histamine-stimulated acid gastric secretion from 'starved' dogs. The figures are averages of the total output of free acid in m-equiv/15 min from two consecutive 15 min collection periods. The percentage fall in acid output refers to the relationship between the lowest acid output reached and the previous maximum value. The half-hour time intervals refer to the start of the histamine infusion as zero

		Histamine 5 μg/kg/min intravenously								
		Time after start of histamine (hr)								
Expt.	Dog wt. (kg)	0·5	1	1·5	2	2·5	3	3·5	4	Fall in acid output (%)
1	15	0·03	0·42	0·95	1·11	1·20	1·28	1·16	—	9
2	24	0·92	3·84	4·49	4·46	4·48	—	—	—	1
3	11	0·04	1·09	5·01	5·45	6·08	5·10	—	—	15
4	15	0·39	2·34	3·03	2·46	2·64	2·75	2·76	2·75	18
5	12	0·17	1·14	1·41	1·55	1·60	—	—	—	0
6	9	0·02	0·32	0·58	0·68	0·94	0·99	0·78	—	21
7	17	0·03	1·25	2·41	2·42	2·42	—	—	—	0
8	18	0·04	1·08	2·18	3·88	4·90	3·80	—	—	22
9	5	0·05	0·56	0·59	1·03	1·21	1·00	—	—	17
10	13	0·05	0·63	1·50	1·66	1·31	—	—	—	21

Histamine-stimulated acid gastric secretion in 'fed' dogs

The results of nine experiments using 'fed' dogs are shown in Table 2. As in the previous series no acid gastric juice was secreted during the period before the start of the histamine infusion. In all experiments acid gastric secretion began within 0·5 hr of starting the histamine infusion and increased rapidly for 1 hr. Between 1 and 2 hr after the start of the histamine infusion, the output of acid fell in all experiments. This fall in acid output became very marked between 2 and 3 hr, and no gastric juice was secreted in any of the experiments between 3 and 3·5 hr after the start of the continuous histamine infusion. The secretion of an acid gastric juice did not return in two experiments which were continued for 4 hr.

TABLE 2. Histamine-stimulated acid gastric secretion from 'fed' dogs. Conventions as in Table 1

		Histamine 5 μg/kg/min intravenously Time after start of histamine (hr)								
Expt.	Dog wt. (kg.)	0·5	1	1·5	2	2·5	3	3·5	4	Fall in acid output (%)
1	21	0·85	2·68	1·53	0·92	0·43	0·00	0·00	—	100
2	20	0·02	0·34	1·10	0·78	0·19	0·00	—	—	100
3	15	0·04	2·00	1·78	0·06	0·10	0·01	0·00	—	100
4	14	0·01	0·05	1·11	0·31	0·17	0·00	—	—	100
5	18	0·15	0·52	1·25	0·41	0·04	0·02	0·00	0·00	100
6	15	0·80	1·42	1·56	0·95	0·09	0·04	0·00	—	100
7	23	0·63	5·12	4·20	1·77	0·18	0·03	0·00	0·00	100
8	11	0·18	0·93	2·14	0·73	0·16	0·09	0·00	—	100
9	14	0·01	0·99	0·87	0·52	0·56	0·11	0·00	—	100

Effects of bilateral cervical vagotomy

The experiments were carried out on 'fed' dogs. Bilateral cervical vagotomy was carried out in four experiments as part of the operative procedures preceding the start of the histamine infusion. The results are shown in Table 3. In these experiments the output of acid increased rapidly during the first 1·5 hr of the histamine infusion and then approximated to a steady state between 1·5 and 2·5 hr. The pattern of response to histamine in the 'fed' vagotomized dog thus appears to be similar to that in the 'starved' dog with intact vagi.

In another six experiments, the results of which are shown in Table 4, bilateral cervical vagotomy was carried out during the decrease of histamine-stimulated secretion seen in 'fed' dogs. In the first three of these experiments the secretion had not completely stopped when the vagi were cut. The acid secretion increased in all these experiments (nos. 1–3, in Table 4) after the vagotomy. However, in the remaining three experiments (nos. 4–6, in Table 4) the secretion had stopped before the vagi were cut; there was no significant return of secretion at the end of an hour.

Effects of variation in the site of pyloric ligation

In all the experiments described so far a ligature was applied at the pyloroduodenal junction to prevent contamination of the gastric juice with regurgitated duodenal contents. This ligature, however, did not prevent the acid juice secreted by the body of the stomach from reaching the mucosa of the pyloric antrum. To prevent this contact the tape ligature was tied at the junction between the pyloric antrum and the body of the stomach. At the start of the experiment the position for the ligature could only be estimated; it was checked post-mortem. As judged by the different appearances of antral and body mucosa (verified histologically in one experiment), the ligature had been applied very close to the junction between body and antrum in four experiments: in another four experiments the ligature had been applied to the antral side of the junction. The results of these eight experiments on 'fed' dogs are shown in Table 5.

In the four experiments where the ligature lay on the antral side of the antral–body junction a marked decrease in acid secretion occurred between

TABLE 3. Effects of bilateral cervical vagotomy on histamine-stimulated acid gastric secretion from 'fed' dogs; conventions as in Table 1. The vagi were cut before the start of the histamine infusion

		Histamine 5 μg/kg/min intravenously								
		Time after start of histamine (hr)								
Expt.	Dog wt. (kg)	0·5	1	1·5	2	2·5	3	3·5	4	Fall in acid output (%)
1	12	0·39	0·48	1·03	1·23	1·20	1·20	—	—	1
2	14	0·22	0·95	2·3	0·35	3·06	—	—	—	0
3	20	0·75	4·55	5·1	4·97	4·90	—	—	—	4
4	11	0·155	0·57	0·51	0·44	0·46	—	—	—	13

TABLE 4. Effects of bilateral cervical vagotomy on histamine-stimulated acid secretion from 'fed' dogs. The vagi were cut during histamine infusion between 2·5 and 3 hr after the start of the histamine infusions. Conventions as in Table 1

		Histamine 5 μg/kg/min intravenously										
		Time after start of histamine (hr)										
Expt.	Dog wt. (kg)	0·5	1	1·5	2	2·5		3		3·5	4	Fall in acid output before vagotomy (%)
1	15	3·21	6·20	5·18	3·68	3·20	Vagotomy	3·40		4·61	5·23	48
2	8	0·20	1·91	2·77	1·72	0·49		1·05		1·20	—	82
3	19	0·15	1·24	1·21	0·73	0·06		0·13		0·98	1·03	95
4	10	0·7	1·40	1·03	0·08	0·03		0·00	Vagotomy	0·01	0·01	100
5	12	0·12	0·76	1·14	0·73	0·06		0·00		0·00	0·00	100
6	18	0·02	0·82	0·86	0·36	0·21		0·01		0·02	0·04	96

TABLE 5. Effect of the site of the gastric ligature on histamine-stimulated acid gastric secretion in 'fed' dogs. Site of ligature: *A*, ligature tied in the middle of the pyloric antrum; *B*, ligature tied at the antral-body junction. Conventions as in Table 1

			Histamine 5 μg/kg/min intravenously						
			Time after start of histamine (hr)						
Expt.	Dog wt. (kg)	Site of ligature	0·5	1	1·5	2	2·5	3	Fall in acid output (%)
1	10	*A*	0·04	2·58	1·63	0·26	0·03	0·00	100
2	13	*A*	0·06	1·72	2·67	1·98	0·18	0·00	100
3	15	*A*	0·30	1·40	1·21	0·23	0·00	0·00	100
4	18	*A*	0·20	0·42	1·68	0·24	0·04	0·00	100
5	16	*B*	0·53	1·90	2·40	2·65	2·90	2·85	2
6	12	*B*	0·01	2·13	3·60	3·97	3·61	3·96	9
7	20	*B*	0·57	2·86	3·65	3·95	2·29	—	42
8	22	*B*	0·08	1·33	1·69	1·78	2·24	2·06	8

1·5 and 2·5 hr. By contrast, in the other four experiments, with the ligature close to the antral–body junction the secretion was maintained at relatively high levels between 1·5 and 2·5 hr, although a fall in secretion can be seen in Expt. 3 at 2·5 hr. This type of maintained secretion is typical of 'starved' dogs.

DISCUSSION

When dogs are used for studies of gastric secretion it is, so far as one can gather from the literature, the almost universal practice to withhold food for from 18 to 36 hr before the experiment. Our results show that the gastric secretory response to histamine in dogs anaesthetized with chloralose is modified by omitting this period of starvation.

The altered secretory response to histamine was obtained only when the vagi were intact and when the continuity of the body with the antrum of the stomach was not disturbed. Thus the initial experimental situation may well determine the nature of the subsequent response to histamine. This phenomenon may not appear in acute or chronic experiments involving denervated stomachs or pouches.

It is more difficult to assess the significance of variations in the site of gastric ligation. The ligature was changed from the pyloro–duodenal junction to the antral–body junction on the assumption that the spread of acid to the antrum might be an essential factor in the altered response to histamine. This suggestion was based on the evidence that the modified histamine response was only evident after the output of acid had reached a high level and that it was vagus-dependent. Since the spread of acid to the antrum is common to both 'fed' and 'starved' dogs, some change in antral sensitivity to acid after feeding is of necessity implied in this suggestion.

SUMMARY

1. A constant intravenous infusion of histamine was used to stimulate gastric acid secretion in dogs with vagi intact, anaesthetized with chloralose and urethane, and prepared with a simple stomach fistula and a ligature at the pyloro–duodenal junction.

2. When the animals had been starved for 24–36 hr before operation, the histamine-stimulated secretion reached a steady state between 1·5 and 3·5 hr after starting the histamine infusion.

3. When the animals were not starved before the experiment, the acid output began to decline after 1·5 hr and had ceased by 3·5 hr after starting the histamine infusion.

4. This decline and cessation of histamine-stimulated secretion in recently fed dogs could be prevented either by bilateral cervical vagotomy or by tying a ligature at the antral–body junction.

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THE EFFECTS OF 5-HYDROXYTRYPTAMINE ON GASTRIC SECRETION IN ANAESTHETIZED DOGS

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In many species, 5-hydroxytryptamine (5-HT) has been found in highest concentration in the walls of the alimentary tract (Erspamer, 1954). 5-HT stimulates some types of smooth muscle directly and it has been suggested that this substance may have a role in gastro-intestinal function as a motor hormone. Feldberg & Toh (1953) found that, in the dog, 5-HT was present in highest concentration in the mucosa of the pyloric antrum and duodenum. Its localization to the mucosa, and in particular its distribution in the pyloric and duodenal areas, suggests a possible relationship to secretory activities in this region of the alimentary tract, since the distribution resembles that described for gastrin and enterogastrone. In contrast to the distribution of 5-HT, histamine, though present in the pyloric antrum and duodenum, is present in higher concentration in the mucosa of the body of the stomach (Douglas, Feldberg, Paton & Schachter, 1951). Schachter, Smith & Toh (1953) gave 5-HT intravenously to cats and dogs but found that it did not elicit an acid gastric juice.

The physiological roles ascribed to 5-HT are legion. It has been regarded as a substance involved in the regulation of systemic blood pressure (Page & McCubbin, 1953). Erspamer & Ottolenghi (1953) believe that it may influence the renal regulation of water balance. Woolley & Shaw (1954) think it plays a part in cerebral function and in normal mental processes. Erspamer & Asero (1952) have put forward the idea that 5-HT is the specific hormone of the enterochromaffin cell system, or argentaffin cells: these are chiefly found in the alimentary tract. Toh (1954) showed that 5-HT is continuously produced from the stomach wall, presumably from the argentaffin cells at this site, when the stomach is perfused *in situ*: in the intact animal the concentration of this substance in the portal blood is always higher than in any other part of the vascular system. It seems probable, therefore, that 5-HT has some relation to gastro-intestinal function. We have examined further the possibility that 5-HT may influence gastric secretion. A preliminary report of the results has already been made (Black, Fisher & Smith, 1956).

METHODS

The experiments were carried out on dogs anaesthetized with chloralose 2% (w/v) and urethane 20% (w/v) 2 ml./kg given intravenously. The animals were starved for 24–36 hr before starting an experiment. The methods are described by Black, Fisher & Smith (1958). Histamine and 5-HT were given into the femoral veins by infusion controlled by a Palmer's Slow Injection Apparatus. The gastric cannula, made from glass tubing with a bore of 2·5 cm, was inserted into the most dependent part of the greater curvature. It had a flange near the inserted end over which a retaining purse-string suture was tied. The inserted end was covered by a perforated glass dome, designed to prevent prolapse of the mucous membrane or blockage by mucus. The abdominal wall was closed round the flange and the animal was placed on a frame to allow drainage of gastric juice. The secretion from the cannula was collected in a funnel where it was filtered through gauze. The gauze was changed every 15 min and by weighing it before and after a rough estimate of mucus output was obtained. The filtrate was collected in a beaker and the volume measured every 15 min. The juice was titrated with Töpfer's reagent as indicator and the acidity expressed in m-equiv/l. The results in the tables are given as averages of consecutive samples, each sample having been calculated as total output in m-equiv/15 min collection period.

RESULTS

Effects of 5-*HT alone on gastric secretion*

Intravenous infusions of 5-HT alone, for periods of 45–90 min, were given to four dogs. 5-HT did not stimulate an acid gastric secretion in any of the experiments. On the other hand, the infusion was accompanied by the production of an alkaline juice rich in mucus. The amounts of this thick secretion expelled from the cannula varied; in one experiment it was as much as 47 ml. when 5-HT had been infused for 45 min at a concentration of 10 μg/kg/min. When a smaller amount of mucoid secretion was expelled from the cannula, there was, at post-mortem, a collection of thick, clear, mucoid, secretion pooling over the area of the pyloric mucosa and duodenum. As much as 45 ml. of mucoid secretion has been collected from the pyloric region at the end of an experiment. The volume of the mucous secretion produced in these experiments was always greater than that found in four control dogs given anaesthetic alone for a similar period. At post-mortem, after infusions of 5-HT alone, the mucosa of the stomach and intestine was very pale.

Effects of 5-*HT on histamine-stimulated gastric secretion*

Infused histamine alone, in animals starved over a period of at least 24 hr, always elicited a rising curve of secretion, with the establishment of a secretory plateau approximately 1·5 hr after onset of the infusion (Black *et al.* 1958). When 5-HT was given for a period of 30 min during continuous histamine-stimulated acid gastric secretion the volume and acidity of the juice decreased with a concurrent increase in the amount of mucus produced. A typical example is shown in Fig. 1. In this particular experiment 15 μg/kg/min of 5-HT given for 30 min was associated with complete inhibition of the acid secretion and with an increased expulsion of mucus. In twelve experiments (Table 1), when the infusion of 5-HT was started 1·5–2 hr after the onset of the histamine-stimulated secretion and while the output of acid was still rising, inhibition of secretion was usually apparent in the first 15 min sample after starting the infusion of 5-HT and continued throughout the infusion period. Recovery from the inhibition had usually begun within 60 min of stopping the infusion, though in a number of experiments recovery was slower than this. The experiments in Table 2 show that two consecutive periods of inhibition can be produced by 5-HT.

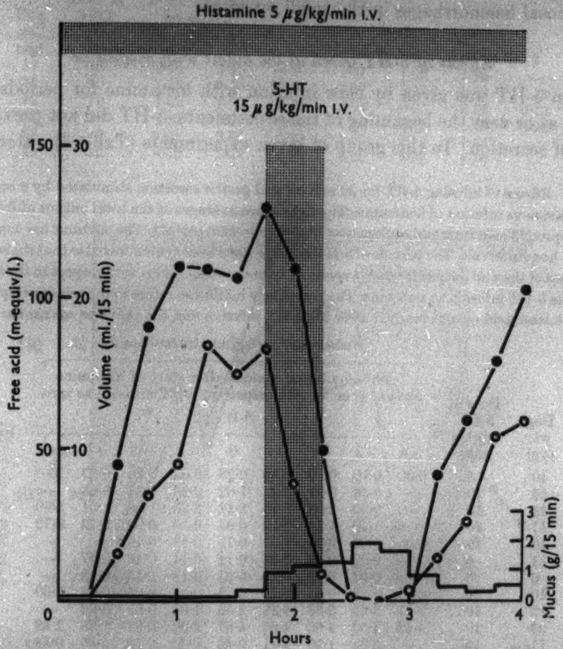

Fig. 1. Effects of infusing 5-HT for 30 min on histamine-stimulated gastric secretion. Ordinates: volume of juice secreted (ml./15 min, open circles) concn. of free acid (m-equiv/l., closed circles), and mucus output (g/15 min, histogram). Horizontal shaded area shows period of histamine infusion and vertical shaded area shows period of 5-HT infusion. It can be seen that the infusion of 5-HT was associated with a fall in both the volume and acidity of gastric juice and a rise in the output of mucus.

In these experiments, where 5-HT had been infused along with histamine, the stomach and intestines were red and engorged at post-mortem. This appearance was indistinguishable from the control dogs which were given histamine alone. In some experiments, where large doses of 5-HT had been given along with histamine, there were small erosions in the gastric mucosa and submucosal haemorrhages in the small intestine.

Effects of 5-HT given at the outset with histamine

When 5-HT was given by slow infusion with histamine for periods of 30-45 min at or near the beginning of the experiments, 5-HT did not prevent the onset of secretion. In this group of seven experiments (Table 3), after initial

TABLE 1. Effects of infusing 5-HT for 30 min on acid gastric secretion stimulated by a continuous intravenous infusion of histamine. The figures are averages of the total output of free acid in m-equiv/15 min from two consecutive 15 min collection periods. The columns are consecutive half-hour intervals. The periods of 5-HT infusion have been synchronized so that the successive values of the acid output for each experiment are shown as + or − with respect to the half-hour of the 5-HT infusion as zero time. The percentage inhibition refers to the relationship between the lowest acid output reached after the 5-HT infusion and the previous maximum value

			Histamine 5 μg/kg/min intravenously									
			Time in half-hour periods after start of histamine shown + or − with respect to 5-HT infusion as zero									
			5-HT									
Expt.	Dog wt. (kg)	Dose of 5-HT (μg/kg/min)	−4	−3	−2	−1	0	+1	+2	+3	+4	Inhibition (%)
1	10	5	0·00	0·10	0·80	0·80	0·28	0·02	0·16	0·57	—	75
2	16	10	—	0·40	0·91	3·07	1·02	0·85	1·32	0·68	—	73
3	25	10	—	0·20	2·03	3·18	2·12	2·35	1·98	2·31	1·80	33
4	17	10	—	1·40	1·16	1·19	0·94	0·20	0·00	0·72	0·72	100
5	10	10	—	0·00	0·78	1·73	0·75	0·48	0·72	—	—	72
6	11	5	—	0·02	0·18	0·78	0·12	0·14	0·48	0·57	—	85
7	6	5	—	0·01	0·09	0·28	0·21	0·18	0·15	0·18	—	47
8	10	20	0·02	0·26	0·46	0·78	0·76	0·30	0·06	0·02	0·00	100
9	12	10	3·17	3·64	5·38	4·36	2·50	1·06	0·93	0·92	—	79
10	23	10	4·44	4·50	4·66	4·65	4·37	2·90	0·92	1·28	2·82	81
11	11	15	—	0·03	1·90	1·91	0·49	0·00	0·13	0·66	0·88	100
12	14	10	0·50	2·05	1·52	2·30	1·91	1·07	0·47	0·21	—	91

TABLE 2. The effects of two successive infusions of 5-HT on histamine-stimulated acid gastric secretion. Conventions as in Table 1. The times are half-hour intervals immediately before, during, and after each 5-HT infusion. Intervals between 0·5 and 1 hr separate the two experimental periods

			Histamine 5 μg/kg/min intravenously							
			Time, in half-hour intervals before, during, and after 5-HT infusion							
			5-HT				5-HT			
Expt.	Dog wt. (kg)	Dose of 5-HT (μg/kg/min)	−1	0	+1	Inhibition (%)	−1	0	+1	Inhibition (%)
1	12	10	5·92	2·17	4·0	63	5·86	3·33	3·17	43
2	18	10	1·86	0·52	0·90	72	2·10	0·83	1·01	58

TABLE 3. Effects of 5-HT on histamine-stimulated acid gastric secretion when infused for 45 min along with histamine at the start of an experiment. Figures as in Table 1

			Histamine 5 μg/kg/min intravenously							
			Time, in half-hour intervals, after start of histamine							
			5-HT infusion for 45 min							
Expt.	Dog wt. (kg)	Dose of 5-HT (μg/kg/min)	1	2	3	4	5	6	7	8
1	17	10	2·40	3·25	4·30	1·51	1·23	1·95	—	—
2	15	10	1·33	3·10	3·75	3·42	2·48	1·80	2·18	—
3	12	10	1·06	1·90	0·36	0·06	0·05	0·06	0·18	0·03
4	12	10	1·80	3·26	4·85	4·84	4·09	1·20	0·93	0·01
5	22	5	1·62	3·65	2·24	1·38	2·15	1·45	—	—
6	18	10	0·82	2·24	2·93	2·26	2·32	2·10	—	—

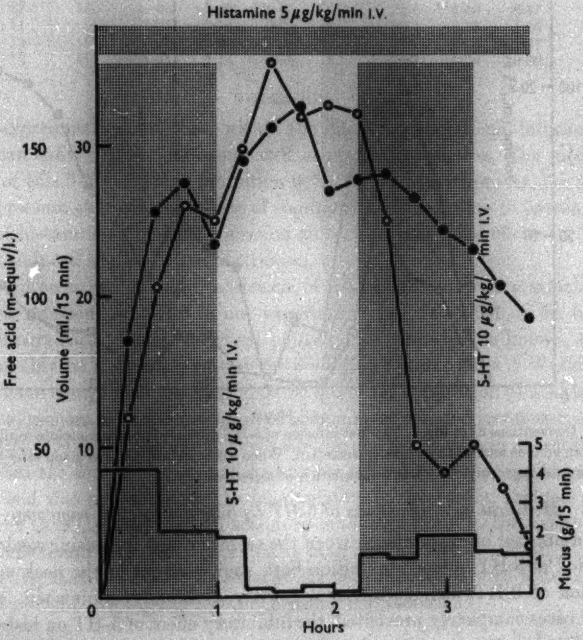

Fig. 2. Conventions in Fig. 1. The infusion of 5-HT given at the outset with histamine had little effect on acid gastric secretion though there were considerable amounts of mucus in the juice. The subsequent infusion of the same amount of 5-HT was associated with marked inhibition of both volume and acidity of secretion and with an increased output of mucus.

stimulation of the acid secretion a fall in acid output occurred between 1 and 2 hr after the end of the 5-HT infusion in all experiments but one. Fig. 2 shows an experiment in which an initial 5-HT infusion did not prevent the onset of histamine-stimulated secretion but a subsequent infusion of 5-HT produced prompt inhibition.

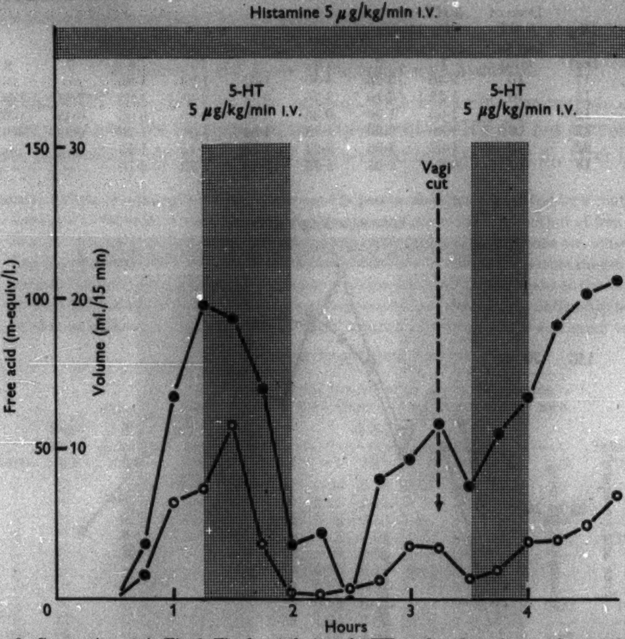

Fig. 3. Conventions as in Fig. 1. The first infusion of 5-HT produced nearly complete inhibition of both volume and acidity of gastric secretion. When a second infusion was given after bilateral cervical vagotomy there was no inhibition of secretion.

Modification of the effects of 5-*HT by bilateral cervical vagotomy*

In a number of experiments, when the secretion was increasing again after a period of 5-HT-induced inhibition both vagi were cut in the neck and the infusion of 5-HT was repeated. Vagotomy invariably diminished, and in many cases completely prevented, the inhibitory effect of 5-HT on histamine-stimulated gastric secretion. A typical experiment is shown in Fig. 3; Table 4 shows examples from nine other experiments. The absence of the inhibitory effect after vagotomy could not be explained by tachyphylaxis since successive injections of 5-HT have similar inhibitory effects on acid gastric secretion stimulated by histamine (Table 2).

TABLE 4. Effects of 5-HT on histamine-stimulated acid gastric secretion when given before and after bilateral cervical vagotomy. Conventions as in Table 1. The times are half-hour intervals immediately before, during and after each 5-HT infusion. Intervals between 0·5 and 1 hr separate the two experimental periods. Histamine was given continuously throughout

			Histamine 5 μg/kg/min intravenously								
			Time, in half-hour intervals, before, during and after 5-HT infusion								
			5-HT					5-HT			
Expt.	Dog wt. (kg)	Dose of 5-HT (μg/kg/min)	−1	0	+1	Inhibition (%)		−1	0	+1	Inhibition (%)
1	23	10	3·83	3·99	1·61	57	Bilateral vagotomy	1·80	2·08	2·09	0
2	10	10	1·25	0·74	0·53	58		0·91	1·00	0·90	1
3	12	10	1·23	1·63	0·47	61		0·81	0·92	0·69	14
4	16	5	3·20	2·77	2·30	28		3·42	3·25	3·51	5
5	10	15	1·14	0·15	0·0	100		0·24	0·47	0·34	0
6	18	10	3·15	0·79	0·77	75		3·80	4·18	3·70	3
7	22	15	4·40	2·40	1·85	57		2·36	2·40	2·47	0
8	11	5	1·03	0·76	0·65	36		0·77	0·86	1·06	0
9	12	10	4·97	2·45	3·28	51		2·97	3·04	2·77	7
10	12	10	1·51	1·14	0·56	63		0·87	0·79	0·98	9

DISCUSSION

These experiments show that, within the described conditions, infusions of 5-HT can inhibit histamine-stimulated acid gastric secretion. The mode of action of this 5-HT-induced inhibition is far from clear. However, the small blood-pressure changes, the effects of vagotomy and the failure to prevent the onset of histamine-stimulated secretion all suggest that these effects of 5-HT are not induced by local vasoconstriction.

It is interesting that the inhibition of acid gastric secretion which has followed infusions of 5-HT in our experiments has depended on an intact vagal innervation. While the vagal pathway for excitation is known, there may possibly be a similar pathway for inhibitory effects. Code & Watkinson (1955) have shown that the vagi must be intact for application of acid to the duodenal mucosa to cause inhibition of histamine-stimulated gastric secretion. Schachter (1949), discussing the acid secretion elicited by certain anaesthetics, notes that the secretion was abolished by the trauma of preparing the gastric fistula and was prevented by the manipulation of the vagi in the neck. He considers that the vagi contain secretory-inhibitory fibres in their supply to the stomach and pancreas, as previously described by Pavlov (1902). It is an odd finding that when 5-HT is given at the beginning along with histamine it fails to prevent the *onset* of histamine-stimulated secretion.

SUMMARY

1. The effects of 5-HT on gastric secretion have been examined in dogs from whom food had been withheld for at least 24 hr. They were anaesthetized with chloralose and urethane and prepared with a stomach fistula and a ligature at the pyloro–duodenal junction.

2. Intravenous infusions of 5-HT alone for 45–90 min did not evoke acid gastric secretion but appeared to increase the production of mucus.

3. When an acid gastric secretion was stimulated by a continuous intravenous infusion of histamine, concurrent infusion of 5-HT for 30 min was found to inhibit the secretion. Recovery from the inhibition had usually begun within 60 min of stopping the 5-HT infusion.

4. Infusion of 5-HT at the start of histamine-stimulated gastric secretion failed to prevent the onset of secretion due to infusion of histamine.

5. When 5-HT was given after bilateral cervical vagotomy it was found to be ineffective in inhibiting histamine-stimulated secretion.

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REVERSIBLE INACTIVATION OF NERVOUS ACTIVITY IN A FISH GUT

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Recent progress in understanding the nature of the movements of the vertebrate alimentary tract has depended to a large extent on studies made on gut muscle freed from ganglia. In mammals three main methods have been employed. In the technique introduced by Magnus (1904) and recently revised by Evans & Schild (1953), most of the enteric ganglion cells are separated from the circular muscle by careful dissection. Apart from the mechanical interference implicit in such a technique, the fact that it is irreversible is a limitation. The second technique involves the use of pharmacological blocking agents such as botulinum toxin, hexamethonium, atropine, 933F, etc. These drugs have been used to inactivate selectively nervous structures present in the gut wall (Feldberg, 1951; Ambache & Lessin, 1955). However, some of these drugs may have some action on the muscle as well as on the nervous elements. Thirdly, Ambache (1946) found that by keeping mammalian intestine for several days at from 0 to 2° C all the nervous structures were permanently inactivated, while the muscle cells recovered on resuspension at body temperature.

This paper shows that the enteric nervous system and motility of the trout gut is essentially similar to that of mammals, with longitudinal myogenic 'pendular' movements and peristaltic waves initiated by stretch stimuli and mediated by the enteric plexuses. In addition there are slow tonus changes and movements under the extrinsic control of vagus and splanchnic nerves.

The brown trout under natural conditions is rarely subjected to temperatures above 16° C and its lethal temperature limit is about 26·5–28° C (Elson, 1942). Evidence is presented below to show that while the activity of the smooth muscle cells remains qualitatively unaffected up to 30° C, most of the nervous elements can be reversibly inactivated by rapidly raising the temperature by only 6° C above normal environmental temperatures (4–16° C).

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METHODS

The gross anatomy of the trout gut from which preparations were taken is shown in Fig. 1. A short muscular oesophagus composed largely of striated muscle fibres opens into the large anterior cardiac portion of the stomach with its prominent longitudinal folds. A shorter, more compact, cylindrical pyloric portion opens through a powerful sphincter into the intestine. About 45 diverticula, the pyloric caeca, branch off the anterior intestine, while the posterior part leads into a muscular rectum.

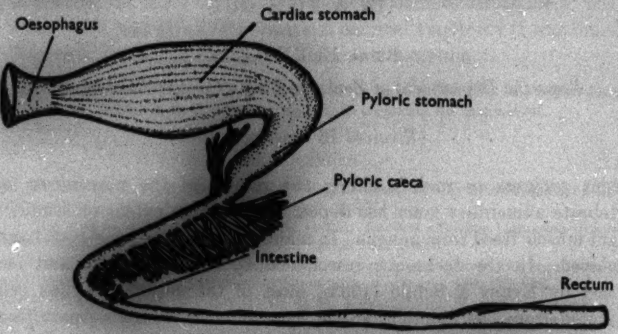


Fig. 1. Diagram of the gross anatomy of the gut of the brown trout.

Isolated preparations of the trout gut were suspended in aerated nutrient saline solution in an organ bath, according to standard pharmacological methods. Several saline solutions were investigated empirically, including that recommended by Young (1932) specifically for fresh-water teleosts, but finally a modified Krebs's solution was found to give the strongest contractions for the longest period of time. The solution consisted of (g/l.): NaCl 5·9; KCl 0·25; $CaCl_2$ 0·28; $NaHCO_3$ 2·1; KH_2PO_4 1·6; $MgSO_4.7H_2O$ 0·29; glucose 2·0.

For recording longitudinal pendular movements cylindrical segments of the stomach, intestine or rectum were suspended with open ends. In some experiments strips of longitudinal muscle were used and suspended under tensions varying from 1 to 4 g.

For recording peristaltic movements, short cylinders of gut were mounted horizontally in the bath. The recording lever was connected with a hook attached to the centre of the preparation at right angles to its main axis, so that contractions registered on the drum were those of the circular muscle.

A nerve–muscle preparation of the stomach was used for stimulation of the vagus nerve *in vitro* and set up under saline solution in a manner based on the technique described by Garry & Gillespie (1955) for the rabbit colon. Over forty such preparations were used in experiments described below. In addition, the vagus nerves to the stomach were stimulated *in situ* in a number of experiments to check the validity of results obtained *in vitro*. Figs. 2 and 3 show the preparation used. The paths of the nerves were traced largely by methylene blue and osmic acid techniques. The main vagus trunks penetrate the gut wall at the oesophagus and run down between the circular and longitudinal muscle coats. Pericellular endings of vagal fibres are found around post-ganglionic enteric neurones in the gut wall. Before the left and right vagus nerves enter the gut wall two or three branches come off each, containing fibres which innervate the striated muscles of the oesophagus directly.

For the preparation the short oesophagus was tied to the bottom of a Perspex arm clamped into a 100 ml. bath, and cotton thread attached to the antrum was connected to a light lever, so that

longitudinal contractions of the stomach were recorded. The vagus nerve was placed over bipolar platinum electrodes encased in an insulated Perspex sheath clamped in the bath. To stimulate the nerve, a rectangular pulse stimulator was used giving negative pulses, the frequency, duration and strength of which was variable. In most cases, 5 sec bursts of electrical pulses were used to stimulate the nerves at intervals of 10 min, so that fatigue effects were avoided.

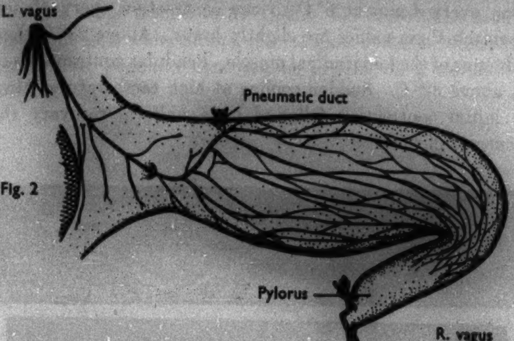


Fig. 2

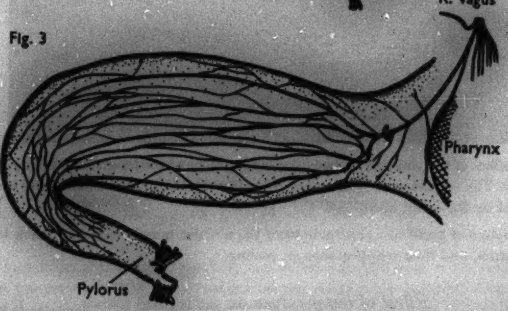


Fig. 3

Fig. 2. Diagram to show the innervation of the trout stomach by the left vagus nerve. The point of entry of the main nerve trunk into the muscle coat is indicated in the diagram. Left dorso-lateral view.

Fig. 3. Diagram to show the innervation of the trout stomach by the right vagus nerve. Right dorso-lateral view.

RESULTS

Effect of temperature on pendular contractions

In the trout, the 'pendular' rhythm consists of slow regular contractions of the longitudinal muscle, approximately one beat every 2 min at normal environmental temperatures, i.e. 4–16° C. These contractions are more marked in the empty gut, but are masked or modified to a large extent by peristaltic waves when parts of the gut are distended with food, particularly in the stomach. There are no pendular contractions in the circular muscle.

The rate of the pendular rhythm is very sensitive to temperature, while the amplitude is affected by mechanical tension as well as by temperature. Pendular contractions show a graded increase in both rate and amplitude from 4 to 30° C (Fig. 4). In the intestine and rectum the rate increases from one contraction every 4 min at 6° C to one contraction every 45 sec at 30° C. In the stomach these values are slightly lower. Above 25° C there is a loss in general tonus of the longitudinal muscle. Pendular contractions cease below 4° C and above 32° C. Sudden cooling at high temperatures always causes a strong initial contraction followed by a uniform recovery rhythm and general increase in tonus.

Fig. 4. The effect of temperature on pendular contractions of the longitudinal muscle of the trout intestine. The temperature is increased from 18 to 32° C and then decreased from the point indicated by the arrow down to 6° C.

Fig. 5. Extinction of peristaltic contractions of the circular muscle of the stomach by raising the temperature, and their reappearance on cooling.

Effect of temperature on peristaltic waves

Peristaltic waves involving a contraction ring of the circular muscle, moving slowly (2 cm/min) aborally, can be seen both *in situ* and *in vitro* in the trout gut maintained at normal environmental temperatures. These movements occur only when the gut is radially extended and each wave is always preceded by a powerful longitudinal contraction.

Unlike myogenic longitudinal pendular activity, peristaltic circular muscle contractions are abolished by rapid heating of the gut (Fig. 5). On cooling the preparation the contractions reappear at about 18° C with increased amplitude. The 'pre-peristaltic' longitudinal contraction is not abolished until a higher temperature is reached, the critical point being more variable (22–26° C). After 18 hr at 0° C, 12 hr at 18° C and 2 hr at 30° C, longitudinal pendular movements return, but peristaltic waves are permanently abolished.

Neither the rate nor the amplitude of circular and longitudinal peristaltic contractions are altered appreciably by increase in temperature, before they are suddenly abolished. In contrast, the rate of longitudinal pendular contractions increases logarithmically with rise of temperature in accordance with both Arrhenius's and Van't Hoff's Laws, the temperature coefficient (Q_{10}) being 2·25 over a range from 6 to 30° C (Fig. 6). Since these laws apply to chemical reactions in a wide variety of biological systems (Sizer, 1943), this result suggests that pendular contractions depend on a metabolic process within the muscle cells.

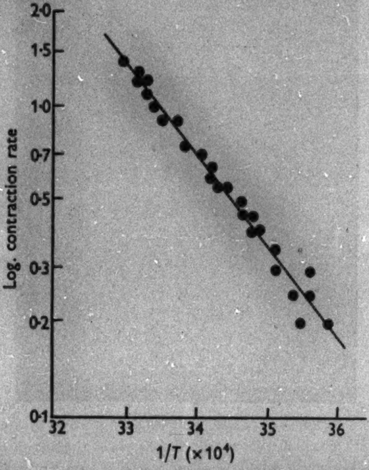


Fig. 6. The logarithm of the rate of pendular contractions plotted against the reciprocal of the absolute temperature. The linear nature of the relationship indicates that Arrhenius's equation is satisfied.

Response of the stomach to vagal stimulation at normal temperatures

Stimulation of either vagus nerve in the trout causes strong contraction of the longitudinal muscle coat of the stomach. A response of this type has been obtained in similar experiments on other teleostean fish (Müller & Liljestrand, 1918; Young, 1936; Barrington, 1942). Normal spontaneous pendular movements return immediately, unless the stimulus is applied for more than 30 sec. If, however, peristaltic waves are present, the circular muscle contractions are inhibited for a period of about 30 min after stimulation of the vagus nerve.

There are characteristically three parts to the response (Fig. 7). The first phase (X) involves an almost instantaneous contraction of the striated oesophageal muscles which remain in a tetanic state until the current is stopped.

There then follows a short relaxation phase (Y), during which the tonus of the muscle often drops below the level maintained during spontaneous activity. At 12° C, approximately 20 sec after the beginning of the stimulation, the main powerful contraction of the smooth longitudinal muscle of the stomach occurs (Z). The characteristic form of the response remains unaltered by changes in the frequency and duration of the pulse and of the strength of the electrical stimulus applied.

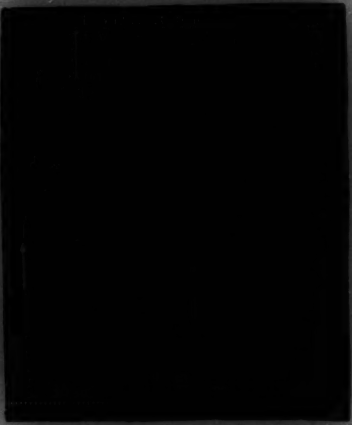
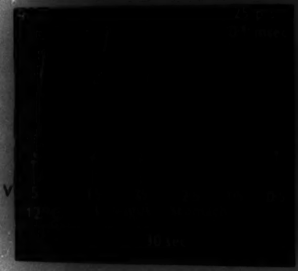


Fig. 7. The response of the trout oesophageal and stomach muscles to stimulation *in vitro* of the vagus nerve at 12° C. The first trace indicates the form of the response seen in all the experiments recorded in this paper where the drum speed is slow (indicated by the 30 sec time markings). The second figure shows the same response specially recorded on a faster drum so that both the duration of stimulation (Stim.) and the detailed form (X, Y and Z) of the contractions can be examined. Stimulating pulse 5 V, frequency 25 p/s, duration 0·1 msec.

Variation of frequency of the stimulating pulse affects the magnitude but not the form of the response (Fig. 8). The maximum amplitude of contraction occurs with a frequency of 25 pulses/sec when the pulse duration is 1 msec. However, it is impossible to divorce consideration of frequency from that of duration of stimulating pulses when examining the magnitude of responses. Variation in voltage of the stimulating current produces contractions of much the same amplitude between 1·5 and 25 V (Fig. 9). No response at all is obtained below 1·5 V. At 25 pulses/sec the largest response was obtained with pulse durations of between 0·01 and 0·1 msec (Fig. 10).

Stimulation of the vagus nerve while the stomach is in different states of tonus has little effect on the nature of the response. However, the magnitude of the contraction is usually less in stomachs with high general tone or if the stimulus is applied at the peak of spontaneous contractions. Conversely, it is greater in stomachs of low general tone or if stimulated during a relaxation phase.

These results hold also for preparations *in situ*, when empty stomachs with high tone give a response of smaller magnitude than do full stomachs with low tone.



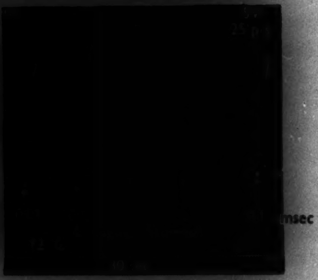


Fig. 8. The response of the trout stomach kept at 12° C to stimulation of the vagus nerve by pulses of *frequencies* ranging from 1 to 1000 p/s. With pulse strength 5 V and duration 1 msec the optimal response is obtained at a frequency of 25 p/s.

Fig. 9. The response of the trout stomach kept at 12° C to stimulation of the vagus nerve by different stimulus *strengths*. With pulse frequency 25 p/s and duration 0·1 msec a response is produced by stimuli ranging from 25 V down to a minimum strength of 1·5 V.

Fig. 10. The response of the trout stomach kept at 12° C to stimulation of the vagus nerve by pulse *durations* varying from 0·01 to 10 msec. With pulse strength 5 V and frequency 25 p/s maximal response is obtained at 0·1 msec.

Effect of temperature on the response of the stomach to vagal stimulation

The response of the smooth muscles of the stomach to stimulation of the vagus nerves can be reversibly abolished by raising the temperature by 6° C at a rate of more than 1° C every 4 min. Thus in Fig. 11 after the temperature is raised from 12 to 18° C in 20 min there is no response to vagal stimulation at *S*. On cooling the preparation the response is re-established, usually with greater magnitude.

There is no critical temperature at which the response always fails. The significant requirement for block is a 6° C rise of temperature in a limited time. The response is eliminated equally well by raising the temperature from 12 to 18° C, from 14 to 20° C or from 16 to 22° C. The response of the stomach to vagal stimulation can be abolished for over an hour by this method (Fig. 12), only the contractions of the oesophageal muscles (phase X) appearing on the

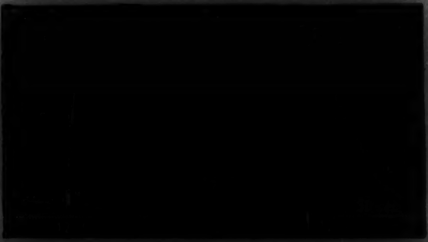


Fig. 11. The effect on the response of the stomach to vagal stimulation of raising the temperature *quickly* (1° C/3 min). The response is abolished after a rise of 6° C (12→18° C) but reappears with greater magnitude on cooling.

Fig. 12. Elimination by warming of the response of the stomach to vagal stimulation for over an hour. The contractions of the striated oesophageal muscles are not abolished by the rise of temperature.

Fig. 13. The effect on the response of the stomach to vagal stimulation of raising the temperature *slowly* (1° C/5 min). The response shows a graded loss in amplitude over a wide range of temperature (16–26° C).

trace during this period. By raising the temperature more slowly (1° C every 5 min), a graded fall in magnitude of response over a wider range of temperature is exhibited (Fig. 13). By raising the temperature of the preparation very slowly (1° C/hr), a response to vagal stimulation has been obtained even at 27° C.

Contractions of the longitudinal stomach muscles due to vagal stimulation are permanently abolished if the preparation is kept at 30° C for over 2 hr, a result similar to that found for peristaltic activity. Preparations kept at 0° C for 18 hr still exhibit vagal responses at 12° C, although the latent period of contraction is unusually long (about 40 sec). The latent period of the vagal response varies considerably with temperature. At 6° C it is about 30 sec; at 12° C, 20 sec; and at 20° C it is no more than 5 sec.

In contrast, direct stimulation of the smooth muscles, even after elimination of the vagal response by quickly raising the temperature of the preparation, produces strong contractions. The contractions of the striated oesophageal muscles, which are innervated directly by vagal fibres, are also not abolished by rise of temperature.

DISCUSSION

It is clear that the response of pendular contractions to temperature change is quite different from the effect of temperature on peristalsis and on the reaction of the stomach to vagal stimulation. The pendular contractions seen in the mammalian gut are considered to be myogenic (Evans & Schild, 1953). That this is the case in the trout gut is supported by experiments in which pendular activity persists in preparations where the enteric plexus has been blocked with hexamethonium or high concentrations of nicotine (Burnstock, unpublished). That pendular activity shows a graded response to both slow and fast rises of temperature in accordance with Arrhenius's and Van't Hoff's Laws suggests that the reaction is a metabolic one. It is probable then that the chemical processes controlling spontaneous activity of the muscle cells themselves are altered over a wide range of temperature. Beyond 30° C, some 4° C above the temperature lethal for the animal, these metabolic processes appear to break down.

The peristaltic wave seen in the mammalian gut is considered by most workers to be under nervous control (Trendelenburg, 1917; Bozler, 1949; Evans & Schild, 1953; Kosterlitz, Pirie & Robinson, 1956; Bülbring, Lin & Schofield, 1958). Evidence that in the trout these movements are neurogenic is already provided by the manner in which peristalsis and contractions of the stomach in response to vagal stimulation cease abruptly after a rapid temperature rise of 6° C; failure of the muscle itself is not involved, since direct electrical stimulation is still effective in these circumstances. It has further been found (Burnstock, unpublished) that nicotine and hexamethonium abolish both peristalsis and the vagal response. It appears, therefore, that the block produced by heat may be exerted at the nerve–muscle junction, the post-ganglionic fibres, or the ganglionic synapses.

Previous workers have suggested that synaptic transmission in mammals is more sensitive to fall of temperature than the post-ganglionic neurone (Eve, 1900; Ambache, 1946; Gillespie & Wishart, 1957). It may be that increase in temperature of the trout gut has a similar blocking action on the synapses. Stimulation of post-ganglionic neurones by nicotine before and after temperature blockage might clarify this point.

The rate of rise of temperature is very critical, suggesting a reversible shock action rather than the existence of an absolute threshold of blockage. In guts acclimatized at 12, 14 and 16° C, vagal-induced contractions are abolished at 18, 20 and 22° C respectively.

Kosterlitz & Robinson (1956, 1957), found that gradual lowering of the temperature of isolated guinea-pig ileum first inhibited the 'emptying phase' of peristalsis, i.e. circular muscle contractions, and then at a lower temperature the 'preparatory phase', i.e. longitudinal muscle contractions, was abolished. Raising the temperature of trout gut gives a comparable order of events. The circular muscle contractions acting through post-ganglionic neurones are abolished by an exactly similar rise of temperature as is necessary to inactivate the response to vagal stimulation. Longitudinal peristaltic contractions, however, are abolished at a higher temperature, although not as high as that necessary to abolish spontaneous muscular contractions. Thus the order of blockage as a result of fast rise of temperature suggested by these results is: (1) synapses or post-ganglionic neurones; (2) neuromuscular junctions; and (3) muscle cells.

This simple method of reversibly inactivating some of the nervous elements contained in the gut wall may serve to obtain more information regarding the part played by nerves in vertebrate gut motility.

SUMMARY

1. A method is described of reversibly abolishing nervous activity exhibited by the trout gut, by raising the temperature at a rate of not less than 1° C every 4 min.

2. Myogenic 'pendular' contractions show a graded increase in both amplitude and rate with temperature changes from 4 to 30° C, in accordance with Arrhenius's and Van't Hoff's Laws.

3. Peristaltic circular muscle contractions are reversibly inactivated at 18° C after a fast rise of temperature.

4. Contraction of the longitudinal stomach muscles induced by electrical stimulation of the vagus nerve can be reversibly abolished for over an hour by a rapid 6° C rise in temperature.

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STUDIES ON THE RESPONSES OF THE ISOLATED NICTITATING MEMBRANE OF THE CAT

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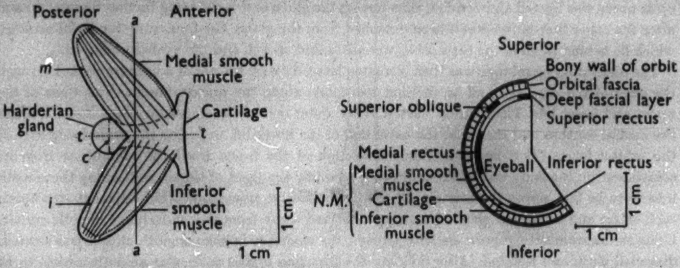
The contraction of the cat's nictitating membrane has long been used experimentally as a sign of activity of the cells of the superior cervical ganglion and for the study of the properties of smooth muscle. There are, however, two major difficulties attending the use of the nictitating membrane in the whole animal. It is influenced not only by its nerve supply, but also by substances released elsewhere and carried by the blood to the membrane; and it is anatomically connected to the external ocular muscles whose contractions may be transmitted to the membrane (Rosenblueth & Bard, 1932; Paton & Thompson, 1953). These difficulties can be overcome by suitable control experiments, but a really rigorous investigation of the smooth muscle retracting the membrane would be greatly helped by its study in isolation, free from humoral and other influences.

The first successful attempt to obtain contractions of the isolated nictitating membrane was made by Westcott & Christensen (1951) using the inferior smooth muscle bundle in the cat. Later, Cervoni & West (1955), Cervoni, West & Fink (1956), and Westcott, Christensen & Marrazzi (1956) used this preparation to study the specificity of the response to acetylcholine. These studies dealt only with the inferior muscle and were relatively restricted. The present paper describes the method whereby a preparation sensitive to appropriate drugs is obtained. The responses of both the medial and inferior muscle bundles of the normal membrane to stimulant drugs and some antagonists are described. The preparation was demonstrated to the Physiological Society in 1955 (Thompson, 1955).

METHODS

Preparations were made from adult cats which had either been (1) anaesthetized with ether, (2) anaesthetized with ethyl chloride and ether followed by chloralose (80 mg/kg I.V.) or (3) subjected to section of the spinal cord at C3 after being anaesthetized with ethyl chloride and ether. The animal was then ready for dissection, which occupied about 1–1·5 hr. With method (3) the membrane was removed at a time when virtually all anaesthetic had disappeared from the tissues. In the figures the method used in preparing the cat for dissection is given in the appropriate legend as 'ether', 'chloralose' or 'spinal cat'.

The cat's nictitating membrane consists essentially of two thin sheets of smooth muscle which are inserted into the adjacent sides of a T-shaped piece of cartilage (Text-fig. 1 *a*). The latter, covered on both its conjunctival and palpebral surfaces by mucous membrane which is reflected back at the corresponding fornices to be continuous with the global and palpebral conjunctivae, respectively, forms the externally obvious 'membrane', whose curved free edge rides over the anterior aspect of the eyeball (see Pl. 1). The cartilage was described by Stibbe (1928) who likened it to a 'T-shaped tool for wiping windows'. As Acheson (1938) has shown, the smooth muscles of the membrane, which he named the medial and inferior muscles, arise deeply in the orbit from the fascial coverings of the medial and inferior recti muscles respectively. Thus the structure of the nictitating membrane is far more extensive than its superficial appearance suggests, and Pl. 1 shows the relationship between the front of the cat's eye and the membrane in various positions, both before and after dissection of the cartilage and smooth muscles. Even when the membrane is fully across the eye, which is not seen under physiological conditions, the majority of the smooth muscle lies deeply in the orbit and the bulk of the tissue lying between the open lids is only cartilage and harderian gland, covered by mucous membrane.



(*a*) (*b*)

Text-fig. 1. *a*, diagram showing the arrangement of the cartilage, harderian gland and smooth muscles of the cat's left nictitating membrane, as seen from the medial aspect when laid out flat. Dotted lines give the approximate areas of cartilage and muscle used when setting up the medial (*m*) or inferior (*i*) preparation; *t*...*t*, line of incision through cartilage; and *c*, extent of cartilage beneath gland. *b*, diagram showing the main anatomical relationships of the nictitating membrane, *NM*, in a coronal section through part of the cat's left orbit, viewed from in front. Both diagrams are on the same scale; the approximate level at which the coronal section, *b*, would pass through the membrane in *a* is shown by the line *a*—*a*: (see also Pl. 1).

From the diagrams of Text-fig. 1 *a* and *b* it can be seen that the structure of the nictitating membrane is butterfly-shaped, and that it normally lies wrapped round the inferomedial side of the eyeball, being sandwiched between the layers containing the orbital fascia externally, and certain of the extra-ocular muscles, internally.

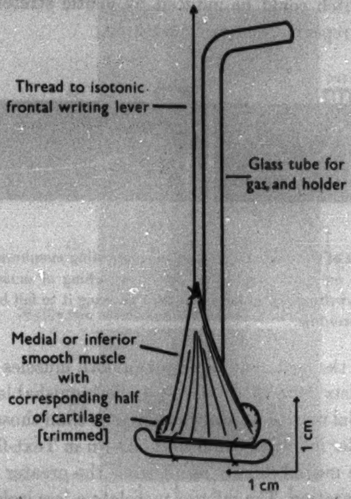
When the experiments were started, a 'butterfly' type of preparation was attempted, the aim being to remove and mount both the medial and inferior muscles still attached to the intervening cartilage, the whole ensemble being arranged in series between a fixed point and the lever thread. Under these conditions, small contractions developed slowly in response to rather large doses of appropriate drugs. These unsatisfactory results were due to the presence of excessive amounts of connective and other tissues inevitable with this type of dissection. Repeated attempts to improve the preparation were unsuccessful and it was, therefore, abandoned.

A sensitive preparation was obtained by three major modifications in technique: (1) preliminary removal of the eyeball and of the surrounding connective tissue; (ii) mobilization of the membrane by dissection of the orbital fascia; and (iii) isolation of either the medial or inferior muscle sheets before removal from the orbit. In detail the procedure was as follows: a lateral skin incision about 1 in. (2·5 cm) in length, beginning at the outer canthus of the eye, was continued posteriorly in the plane of the palpebral fissure. The eyeball, controlled with a ligature which passed through the cornea into the anterior chamber and out again, was retracted medially and after an opening had been made through the conjunctiva the belly of the lateral rectus muscle was isolated, ligated near the globe and cut between the ligature and the eyeball. The other ocular muscles were treated similarly, using different-coloured threads to avoid later mistakes in identification. This is important, because the muscles of the nictitating membrane arise deeply in the orbit from the fascial layers of the medial and inferior recti. Identification of the medial and inferior recti thus greatly assisted in locating the smooth muscles. Identification of the superior and inferior oblique muscles was also useful because the medial smooth muscle lies on the medial side of the superior oblique whilst the superomedial edge of the inferior smooth muscle is crossed by the belly of the inferior oblique. The extra-ocular muscles were isolated and ligated in the following order: lateral rectus, superior oblique, superior rectus, inferior oblique, inferior rectus and medial rectus. After the first two of these extra-ocular muscles had been detached the optic nerve was ligated and divided, thus freeing the globe and facilitating further dissection; and after the other four muscles had been detached from the globe, the four retractor bulbi muscles, which lie behind them in the cat's orbit, were detached as well and the eyeball removed.

The nictitating membrane was then retracted laterally with the aid of a ligature passed through the edge of the cartilage and an incision was made along the mucocutaneous junctions of the upper and lower lids. The incision started at the outer end of the upper lid, went down through the medial canthus and thence to the outer end of the lower lid, exposing the underlying edge of the orbital fascia (Text-fig. 1*b*). Blunt dissection of the fascia freed the membrane from its attachment to the bony wall of the orbit, thus enabling the sheet of tissues containing the smooth muscle to be laid across the now empty orbit. Connective tissue and other adjacent structures, such as fat and harderian gland, were then dissected away from the underlying smooth muscle. Using the anatomical signposts described above, the medial or inferior smooth muscle was located, dissected clean and isolated. After defining the insertion of the particular smooth muscle to be used into the cartilage, the latter was cut in half at right angles to its free edge (Text-fig. 1*a*). The muscle with its attached portion of cartilage was removed and mounted in an organ bath, using a ligature passed through the cartilage for attachment to the glass holder, and a ligature previously placed in position round the opposite end of the muscle for attachment to the writing lever (Text-fig. 2).

The capacity of the organ bath was either 70, 20 or 10 ml., containing Krebs's solution of the following formula: NaCl 6·9, KCl 0·35, $CaCl_2 . 2H_2O$ 0·37, KH_2PO_4 0·16, $MgSO_4 . 7H_2O$ 0·29, glucose 1·0, $NaHCO_3$ 2·1 g/l. The solution was kept at 37° C and O_2 95 % CO_2 5 % was constantly bubbled through it. The pH of the Krebs's solution in the reservoir aspirator at room temperature was 7·3, but in the bath at 37° C it was 7·5. The pH measurements were made with a Pye pH meter. A light isotonic frontal writing lever with load 1 g was used giving ×10 magnification and the contractions were recorded on smoked paper.

The drugs to be examined were added to the bath for a period of 1·5 min, except where otherwise stated: after each test the bath was washed out twice and the lever was allowed to return to the base line. In good preparations the time for full relaxation after small contractions was often as short as 2 min, but after maximal contractions relaxation would often require 45 min. The following drugs were used: acetyl-β-methylcholine as chloride (Mecholyl), acetylcholine as chloride, adrenaline as bitartrate, atropine as sulphate, barium as chloride, calcium as chloride, carbachol as carbamylcholine chloride, cocaine as hydrochloride, decamethonium as iodide, dibenamine as hydrochloride, dibenzyline as hydrochloride, dihydroergotamine as methane-sulphonate, ergotamine as tartrate, eserine as sulphate, hexamethonium as bromide, 5-hydroxytryptamine as creatinine sulphate, hyoscine as bromide, *iso*propylnoradrenaline as sulphate, lysergic acid and its brom-derivative (BOL 148) as diethylamide, morphine as sulphate, neostigmine as methylsulphate, nicotine as hydrogen tartrate, L-noradrenaline as hydrogen tartrate, pilocarpine as nitrate and tetramethylammonium as bromide. All the values given in the text refer to the salts. Potassium was added to the bath as potassium chloride and the final concentration measured in terms of the normal K^+ concentration in the Krebs's solution; thus the concentration was raised by an amount 2, 3 or 4 times that normally present in the bath. Pitocin and Pitressin (Parke, Davis and Co.) were used for oxytocin and vasopressin respectively. Bradykinin was that prepared and kindly supplied as a gift by Miss D. Armstrong. If not otherwise stated, the strength of the drugs used is given as the final concentration in the bath

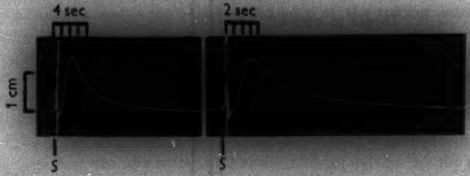

Thread to isotonic frontal writing lever
Glass tube for gas and holder
Medial or inferior smooth muscle with corresponding half of cartilage [trimmed]
1 cm
1 cm

Text-fig. 2. Diagram showing the method of mounting the isolated medial or inferior smooth muscle of the nictitating membrane for recording contractions. The cartilage acts as a convenient fixed point anchored to the glass holder with two ligatures; organ bath not shown

RESULTS

The freshly mounted muscle is usually found to be in a state of contraction from which it will relax if it is left undisturbed. This normally takes about 20–30 min, but with some preparations it may be as long as 2–3 hr before the lever finally stops its gentle descent. Muscles which relax rapidly are likely to give larger and brisker contractions than those which relax slowly. It is necessary to obtain as much relaxation as possible in order to achieve a steady base line. Once this has been achieved, provided sufficient rest periods are allowed after each contraction, the lever usually returns to the original base line. The muscle is susceptible to excessive handling and stretching, and this may result in an erratic and useless preparation. For this reason the relaxation either at the beginning or during the course of an experiment must not be hastened by placing extra loads on the muscle.

Spontaneous activity is usually absent, but occasionally develops after the muscle has been in the bath for several hours. When this happens, the preparation is no longer usable, but sometimes careful adjustment of the oxygen bubbling through the bath reduces the spontaneous activity to a level which renders it again suitable for experiment. The preparations which showed spontaneous activity differed sometimes from those which were quiescent by the fact that a twitch could be induced by gentle stretching of the muscle (Text-fig. 3) or by repeated and rapid washing.



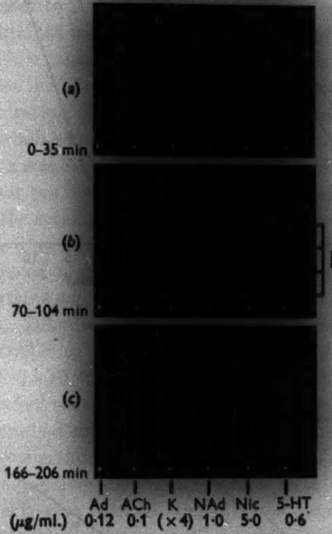
Text-fig. 3. Contraction of the medial muscle of the nictitating membrane (chloralose). Tracings of twitch response on gentle stretching. At *S*, stretching of muscle produced by gently depressing the non-writing end of lever and then allowing it to fall back. Drum speed twice as fast in second recording.

The responses of the medial and inferior smooth muscles to stimulant drugs and their antagonists were qualitatively indistinguishable, but the contractions from the medial muscle were always greater than those recorded from the inferior muscle under identical conditions (shown in Text-fig. 5). The stronger contractions of the medial muscle result from the greater amount of smooth muscle in this preparation. The inferior muscle is a less isolated structure, and on its dissection it is difficult to separate it from the inferior oblique muscle without damage to some of its muscle fibres.

Drugs to which the preparation is sensitive

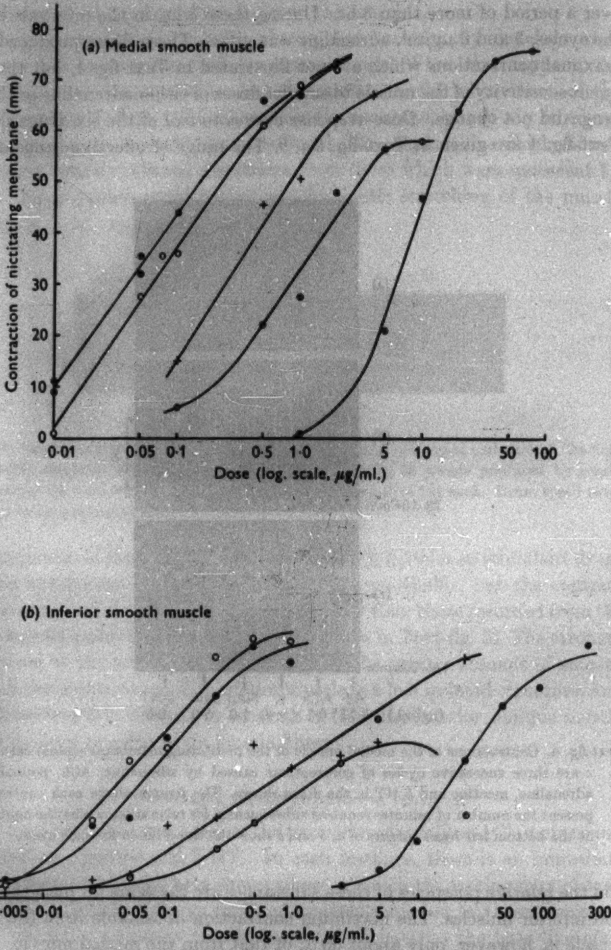
Text-fig. 4 shows the responses of the muscle to adrenaline, ACh, potassium, noradrenaline, nicotine and 5-HT. In each instance, there is an immediate brisk contraction followed by a slower phase in which the tracing may or may not level off. Three successive cycles of contractions due to these drugs are shown; the times necessary for the lever to fall back to the base line after the drug was washed out are given on top of each tracing. Compared with the longitudinal muscle of the guinea-pig ileum, this muscle contracts more slowly and relaxation takes a much longer time. Experiments in which several drugs and antagonists are being used are therefore lengthy; this, however, is offset by the fact that the preparation gives very reproducible results. As shown in

Text-fig. 4, the responses to any one of the six drugs tested varied only slightly over a period of more than 3 hr. During these 3 hr, in the intervals between the cycles, 3 and 6 μg/ml. adrenaline was given. These doses produced nearly maximal contractions which are not illustrated in Text-fig. 4, but the subsequent sensitivity of the muscle to smaller doses of either adrenaline or the other drugs did not change. Dose–response curves to five of the six drugs shown in Text-fig. 4 are given in Text-fig. 5*a*, *b*. The range of effective concentrations



Text-fig. 4. Contractions of the medial muscle of the nictitating membrane (spinal cat). *a*, *b* and *c* are three successive cycles of contractions caused by adrenaline, ACh, potassium, noradrenaline, nicotine and 5-HT in the doses shown. The figures above each contraction represent the number of minutes required subsequently for relaxation, whilst the figures shown at the bottom left-hand corners of *a*, *b* and *c* show the times taken for each cycle.

and the relative potencies of these substances are the same for both the medial and inferior muscles. The maximum contraction obtainable from the inferior muscle is, however, only about 60% of that from the medial muscle. A consistent finding apparent from the dose–response curves of these different drugs is the inability of 5-HT to produce on either muscle a maximum response when given in large doses. In the graph relating to the inferior muscle, the largest dose of 5-HT produced in fact a contraction which was considerably smaller than that produced by a five-times smaller dose.

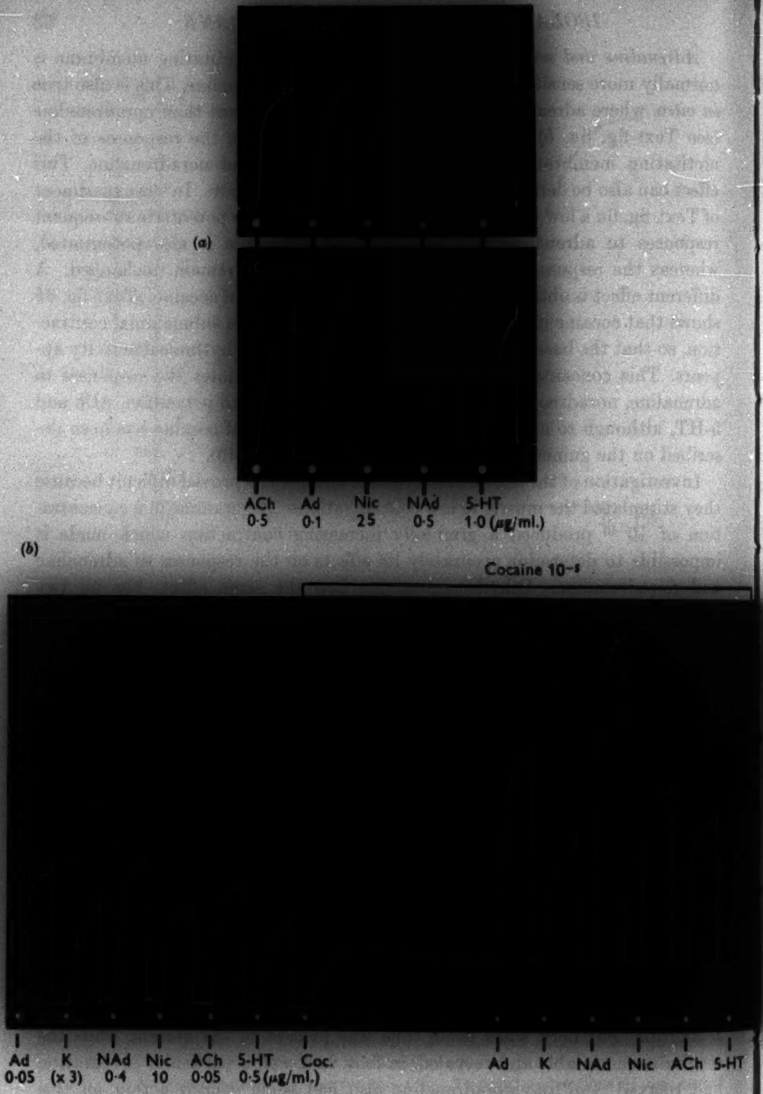


Text-fig. 5. Dose–response curves of contractions of *a* medial and *b* inferior isolated smooth muscles of the nictitating membrane to adrenaline (●), acetylcholine (○), noradrenaline (+), 5-hydroxytryptamine (◎) and nicotine (⊕). Maximal contractions of the medial muscle are larger than those of the inferior. The maximum response to 5-HT is less than that elicited by any of the other drugs. Preparations from two spinal cats; identical recording in both experiments.

Adrenaline and noradrenaline. In vivo, the cat's nictitating membrane is normally more sensitive to adrenaline than to noradrenaline. This is also true *in vitro*, where adrenaline is about five times more potent than noradrenaline (see Text-fig. 5*a*, *b*). Cocaine is known to potentiate the responses of the nictitating membrane *in vivo* to both adrenaline and noradrenaline. This effect can also be demonstrated in the isolated preparation. In the experiment of Text-fig. 6*a* a low concentration (5×10^{-8}) is shown to potentiate subsequent responses to adrenaline and noradrenaline. Nicotine is also potentiated, whereas the responses to ACh, 5-HT and potassium remain unchanged. A different effect is obtained with a higher concentration of cocaine. Text-fig. 6*b* shows that cocaine in a concentration of 10^{-5} produces a submaximal contraction, so that the base line is raised. In addition, small rhythmical activity appears. This concentration of cocaine not only potentiates the responses to adrenaline, noradrenaline and nicotine, but also those to potassium, ACh and 5-HT, although to a smaller degree. A similar effect of cocaine has been described on the guinea-pig ileum by Feldberg & Lin (1949).

Investigation of the action of adrenaline antagonists proved difficult because they stimulated the muscle in low concentrations. Ergotamine in a concentration of 10^{-10} produced a gradually increasing contraction which made it impossible to determine accurately its effects on the responses to adrenaline and noradrenaline. Dihydroergotamine had the same effect, but on two occasions it was possible to block adrenaline and noradrenaline responses by concentrations of $1\text{–}2 \times 10^{-8}$. Dibenamine and dibenzyline, in concentrations which regularly blocked the adrenaline and noradrenaline responses, produced only a very slight gradual contraction. Text-fig. 7 illustrates a typical experiment. A control cycle is shown at *a*. In the presence of dibenamine $1{\cdot}5 \times 10^{-6}$ the responses to adrenaline and noradrenaline were obliterated, those to nicotine and to 5-HT were reduced, and those to potassium and ACh were slightly increased. A concentration of dibenamine 5×10^{-6} produced a slow gradual contraction, which was seen in a rising base line. This concentration of dibenamine not only abolished the adrenaline and noradrenaline contractions, but caused a reversal of the responses; the effect, though small, was a genuine relaxation. The nicotine contraction was greatly reduced, no longer sustained, and occurred after an increased latency. The responses to both 5-HT and potassium were slightly further depressed than with the weaker concentration of dibenamine.

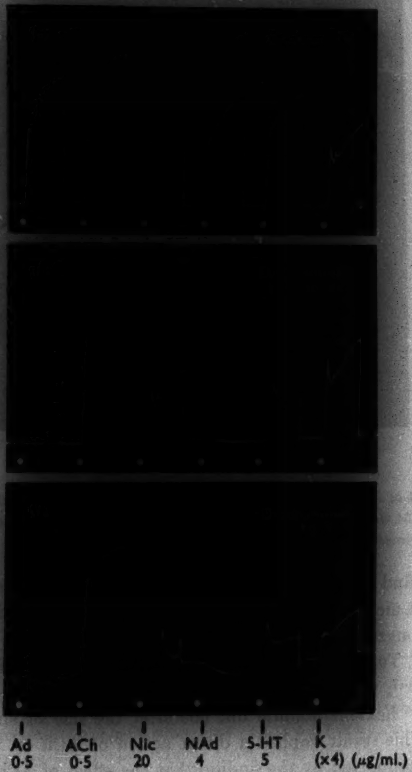
isoPropylnoradrenaline. This drug in concentration of 1 μg/ml. produced relaxation, but only when some tone was present in the muscle. Thus the results were variable and revealed, in fact, how well a particular preparation had relaxed. *iso*Propylnoradrenaline also had some excitor action on this muscle, and these two actions are illustrated in Text-fig. 8. The muscle was partly contracted as a result of the addition of LSD 10^{-9} to the bath. This



Text-fig. 6. *a*, contractions of the inferior muscle of the nictitating membrane (spinal cat), to ACh, adrenaline, nicotine, noradrenaline and 5-HT in the doses indicated; lower tracing in the presence of cocaine 5×10^{-6}; *b*, contractions of the medial muscle of the nictitating membrane (spinal cat); effect of cocaine 10^{-5} on the responses to adrenaline, potassium, noradrenaline, nicotine, ACh and 5-HT.

partial contraction persisted after washing out the LSD, but the muscle relaxed on the addition of *iso*propylnoradrenaline 10 μg/ml. When the same dose was later given to the relaxed muscle after the previous dose of *iso*propylnoradrenaline had been washed out, it caused a contraction. These two kinds of responses have been recorded from several preparations which have been in a state of contraction following the addition of a variety of stimulant drugs to the bath.

Acetylcholine. The sensitivity of the isolated nictitating membrane to ACh is about as great as that to adrenaline, as shown by a comparison of the dose–

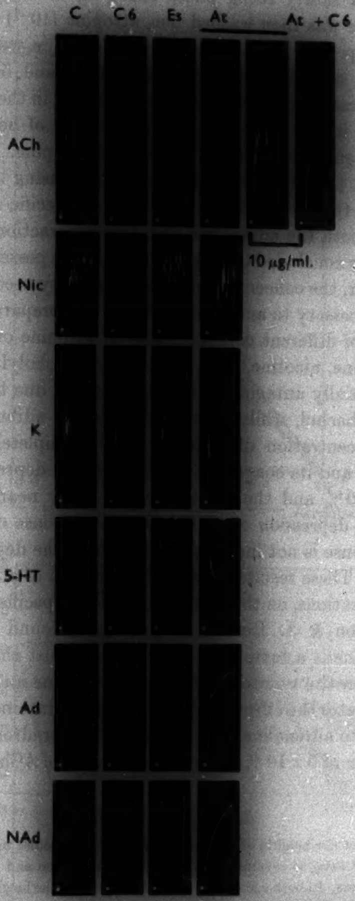


Text-fig. 7. *a*, contractions of the medial muscle of the nictitating membrane (spinal cat), to adrenaline, ACh, nicotine, noradrenaline, 5-HT and potassium, in the doses shown; in the presence of dibenamine, at *b*, $1{\cdot}5\times10^{-6}$; and, at *c*, 5×10^{-6}, respectively. The dibenamine was allowed to act for 45 min before the records at *b* were obtained; the records at *c* obtained immediately after increasing the concentration of dibenamine.

response curve for these two drugs (Text-fig. 5*a*, *b*). The sensitivity to acetyl-β-methylcholine and carbachol is about the same as that to acetylcholine (Text-fig. 10*a*). All three drugs produced contractions very similar in shape. Pilocarpine was about ten times less active than acetylcholine and the muscle contracted more slowly and continued to contract during the whole 1·5 min the pilocarpine was kept in the bath.

Text-fig. 8. Effect of *iso*propylnoradrenaline on the medial muscle of the nictitating membrane (spinal cat). At the onset of the tracing the muscle was partly contracted as the outcome of previous treatment with LSD 10^{-9}: *W* = washing (for details see text).

In order to find out whether the action of ACh was a muscarinic effect, or a nicotinic effect mediated through the activation of nervous tissue or ganglion cells lying amongst the smooth muscle cells, its sensitivity to hexamethonium was examined. Text-fig. 9 shows that the responses were unaffected by hexamethonium 10^{-7}, which abolished the nicotine contraction. Even a concentration of 10^{-4} hexamethonium did not alter the size or shape of the ACh contraction. This result implied a direct action of ACh on the muscle; this conclusion is supported by the finding that the effect of ACh on this muscle, like other muscarinic actions of ACh, is potentiated by eserine and abolished by atropine and hyoscine. In the experiment (Text-fig. 9) the addition of eserine 10^{-6}, after the hexamethonium was washed out and its effect had disappeared, resulted in the immediate potentiation of the ACh contraction, which manifested



Text-fig. 9. Contractions of the medial muscle of the nictitating membrane (spinal cat) to ACh (0·2 µg/ml.), nicotine (6·0 µg/ml.), potassium (× 4 K+), 5-HT (2·0 µg/ml.), adrenaline (0·2 µg/ml.), and noradrenaline (1·0 µg/ml.) from above downwards. The contractions in the first vertical column are controls, those in the second column were obtained in the presence of hexamethonium 10^{-7}, those in the third in the presence of eserine 10^{-6}, and those in the fourth in the presence of atropine 10^{-7}. The two additional contractions in the top right-hand corner are due to a large dose of ACh (10 µg/ml.) in the presence of atropine 10^{-7} alone and with hexamethonium 10^{-7}.

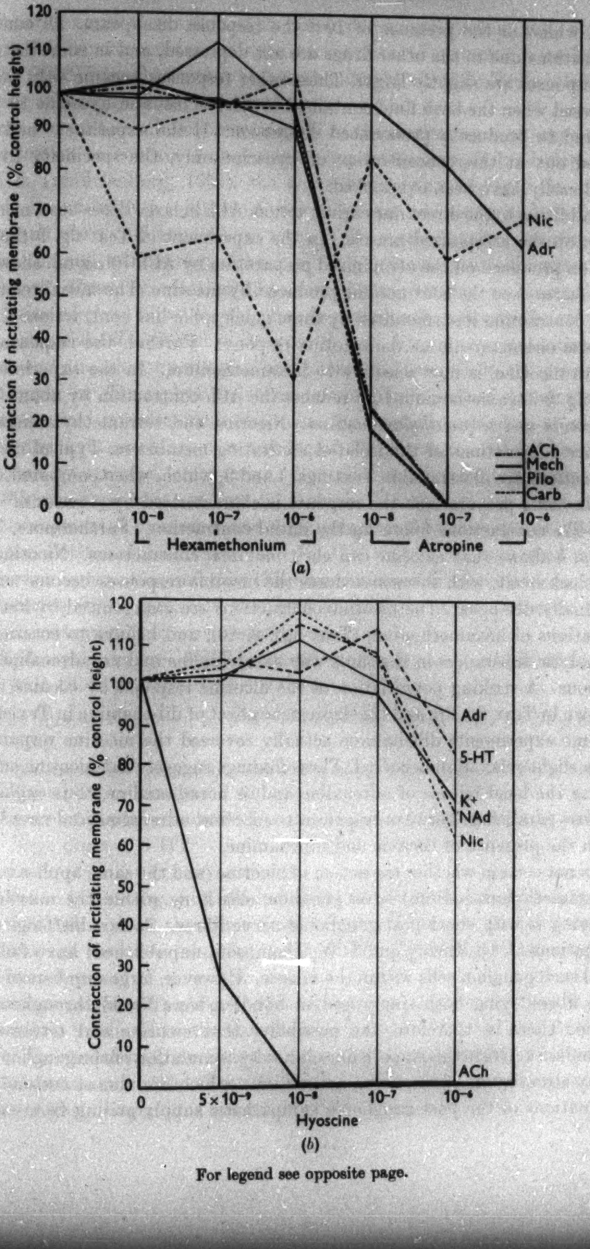
itself mainly by a slowly rising phase following the initial quick contraction. When eserine was then washed out, atropine (10^{-7}) abolished the ACh response. The responses to potassium and adrenaline were scarcely altered by either eserine or atropine; those to nicotine and 5-HT were slightly depressed. The noradrenaline contraction was brisker in the presence of eserine than it had been in the control or in the presence of hexamethonium, but returned to its original shape in the presence of atropine.

Cervoni & West (1955) and Cervoni *et al.* (1956) using the isolated inferior muscle attributed the action of atropine to a non-specific depression, because they found that both the adrenaline and ACh contractions were reduced by this drug, and they concluded that the muscle did not possess specific receptors for ACh. However, the concentration of atropine they used was 10^{-6}, which is 100 times that necessary to antagonize ACh in this preparation. Text-fig. 10*a* shows the effect of different concentrations of atropine on contractions produced by adrenaline, nicotine, ACh, pilocarpine, mecholyl and carbachol. At 10^{-8} atropine partially antagonizes the contractions due to ACh, pilocarpine, mecholyl and carbachol, whilst leaving that due to adrenaline intact; a ten times higher concentration of atropine (10^{-7}) completely antagonizes the responses to ACh and its congeners, but in addition depresses the adrenaline response about 20% and the nicotine response by nearly 40%. Atropine (10^{-6}) produces a depression of the adrenaline response of nearly 40%, but the nicotine response is not increased and, in fact, the depression this time is only about 30%. These results show that the action of atropine, particularly in higher concentrations, on this preparation is not specific.

W. D. M. Paton & J. Rosales (unpublished) found on the guinea-pig ileum that hyoscine is a more specific ACh antagonist than atropine is. The same was found on the isolated nictitating membrane and is shown in Text-fig. 10*b*. It illustrates the effects of different concentrations of hyoscine on the contractions due to adrenaline, 5-HT, potassium, noradrenaline and nicotine. At a concentration of 5×10^{-9}, hyoscine depresses the ACh response by nearly

Legend to Text-fig. 10

Text-fig. 10. Graphs of the heights of the contractions of two medial muscles of the nictitating membrane (spinal cats) to several stimulant drugs both alone and in the presence of three different antagonists. In both *a* and *b* the ordinates represent the heights of the concentrations expressed as a percentage of the control heights, whilst the abscissae represent the contractions of the antagonists used: equivalent doses of drugs were used except in the case of nicotine and pilocarpine, when doses which produced smaller contractions were used. In *a*, responses to ACh 0·1 μg/ml., mecholyl 0·1 μg/ml., pilocarpine 1·0 μg/ml., carbachol 0·1 μg/ml., and nicotine 5 μg/ml. are shown alone and in the presence of first hexamethonium 10^{-8}, 10^{-7} and 10^{-6} and secondly, atropine 10^{-8}, 10^{-7} and 10^{-6}, respectively. In *b*, responses to ACh 0·1 μg/ml., adrenaline 0·25 μg/ml., 5-HT 0·5 μg/ml., potassium $\times 4\ K^+$, noradrenaline 0·5 μg/ml. and nicotine 20 μg/ml. are shown alone and in the presence of hyoscine 5×10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6}.



(*a*)

(*b*)

For legend see opposite page.

75%, whilst in the presence of 10^{-8} the response disappears. In contrast, contractions due to the other drugs are not depressed, and in some instances the responses are slightly larger. These other responses became only slightly depressed when the bath fluid contained 10^{-7} hyoscine, and hyoscine 10^{-6} was required to produce a pronounced depression. If the experiment had been carried out at this concentration of hyoscine only, the specificity to ACh would easily have been overlooked.

In addition to the direct muscarinic action, ACh in large doses has a nicotinic action on the atropinized muscle. In the experiment of Text-fig. 9 the contraction produced on the atropinized preparation by ACh 10 μg/ml. shows the same features as the contractions produced by nicotine. The initial relatively small contraction is surmounted by three quick spike-like contractions similar to those characteristic of the nicotine response. Further, the response, like that to nicotine, is now sensitive to hexamethonium. In the experiment of Text-fig. 9 hexamethonium 10^{-7} reduces the ACh contraction by about 50%.

Nicotine and tetramethylammonium. Nicotine and tetramethylammonium produce contractions of the isolated nictitating membrane. Typical nicotine contractions are illustrated in Text-figs. 4 and 9, which, when compared, show that in some preparations the response is characterized by a series of quick spike-like contractions following the initial contraction. Furthermore, Text-fig. 5*a*, *b* shows that nicotine can elicit maximal contractions. Nicotine will also block itself; with successive doses the nicotine responses become smaller and finally disappear. The nicotine contractions are antagonized by low concentrations of hexamethonium (Text-figs. 9, 10), and behave to cocaine and adrenolytic substances in the same way as adrenaline and noradrenaline contractions. A striking potentiation of the nicotine response by cocaine (10^{-5}) is shown in Text-fig. 6*b*, and the depressant effect of dibenamine in Text-fig. 7. In some experiments dibenamine actually reversed the nicotine response so that a slight relaxation occurred. These findings suggest that nicotine acts by causing the local release of adrenaline and/or noradrenaline, thus explaining the close parallelism between responses to nicotine, adrenaline and noradrenaline in the presence of cocaine and dibenamine.

It is not certain whether the action of nicotine (and the same applies to that of tetramethylammonium) is on ganglion cells lying within the muscle and supplying it with short post-ganglionic nerve fibres. So far, histological investigations (J. G. Murray and J. W. Thompson, unpublished) have failed to reveal such ganglion cells within the muscle. However, large numbers of small nerve fibres, lying both singly and in bundles, were found throughout the muscle. There is, therefore, the possibility that nicotine and tetramethylammonium activate this smooth muscle not by stimulation of the ganglion cells, but by stimulation of these fine nerve fibres, which are almost certainly the terminations of the post-ganglionic sympathetic supply arising from cells in the superior cervical ganglion. This conclusion is not incompatible with the finding that hexamethonium antagonizes the nicotine responses, since Douglas & Gray (1953) have shown that hexamethonium blocks the stimulant action of nicotine on sensory nerve endings.

Since the post-ganglionic nerve supply to the nictitating membrane of the cat is known to contain a cholinergic component (Bacq & Fredericq, 1935; Burn & Trendelenburg, 1954), the nerve fibres lying amongst the smooth muscle are probably a mixture of post-ganglionic adrenergic and cholinergic fibres, and one would expect both types of fibres to be excited by drugs such as nicotine and tetramethylammonium. The attempt to reveal excitation of cholinergic fibres by nicotine or tetramethylammonium was inconclusive. Excitation of these fibres should produce a contraction due to the release of ACh, which should, therefore, be resistant to dibenamine, potentiated by eserine and abolished by atropine and hyoscine. However, in the presence of dibenamine or dibenzyline in concentrations sufficient to antagonize the adrenaline and noradrenaline responses, the remaining response to nicotine or tetramethylammonium was not significantly potentiated by eserine or depressed by atropine, or hyoscine. Nevertheless, the effect of eserine illustrated in the experiment of Text-fig. 9 shows that the nicotine contraction is slightly augmented by eserine, and slightly depressed by atropine. It is possible that the nictitating membrane used for this experiment contained a small cholinergic innervation and that this does not occur in all membranes. Bacq & Fredericq (1935) estimated that only about 50% of cats possessed a cholinergic post-ganglionic nerve supply.

5-*Hydroxytryptamine.* 5-HT produced contractions which differed in three ways from those produced by other drugs:

(1) The muscle contracted by 5-HT relaxed only slowly after washing out the drug.

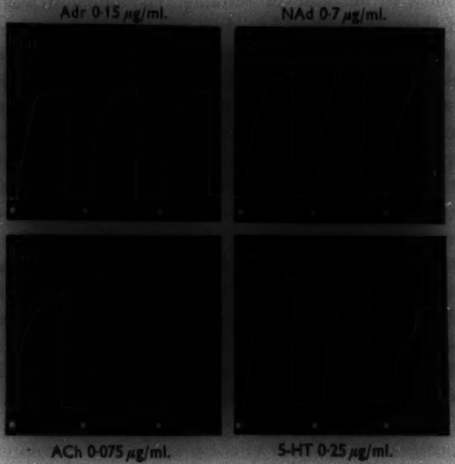
(2) With successive administration of the same amount of 5-HT the contractions became smaller.

(3) It was not possible to obtain maximal contractions of the muscle with even large doses of 5-HT.

As shown in Text-fig. 4, it took a few minutes longer for the muscle to relax after 5-HT than after the other drugs examined. Sometimes the lever continued to rise for a few minutes after the 5-HT had been washed out, before relaxation began.

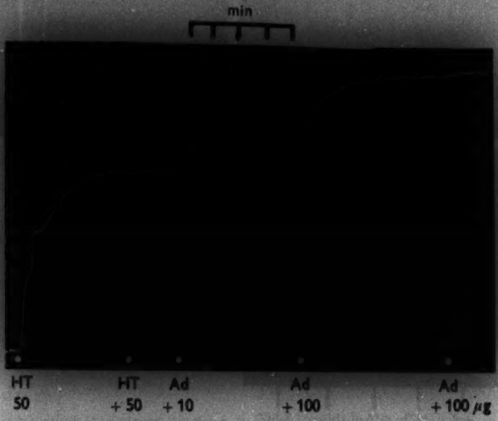
The experiment of Text-fig. 11 illustrates the progressive diminution in the contractions produced by three successive administrations of the same dose of 5-HT. By comparison, successive responses to the same doses of adrenaline, noradrenaline, and ACh (Text-fig. 11*a–c*) either remained unchanged or became slightly larger.

The inability of 5-HT to cause maximal contractions of the nictitating membrane is evident from a comparison of its dose–response curves with that of other drugs, as has been mentioned before. This inability is illustrated in a different way in Text-fig. 12. It shows that the muscle partially contracted by 5-HT does not contract further by adding more 5-HT to the bath, although adrenaline produces an additional contraction. These results show that 5-HT in amounts in excess of those required to produce a maximum contraction for this substance does not fully contract the muscle. Identical results have been obtained in corresponding experiments using either noradrenaline or ACh instead of adrenaline.



Text-fig. 11. Contractions of the medial muscle of the nictitating membrane (ether) to *a* adrenaline, *b* noradrenaline, *c* ACh and *d* 5-HT in the doses shown. In each set of contractions the same dose was repeated twice, the drug being added again as soon as the muscle had recovered from the preceding dose. Each drug was in the bath for 1·5 min; drum speed faster than in other recordings.

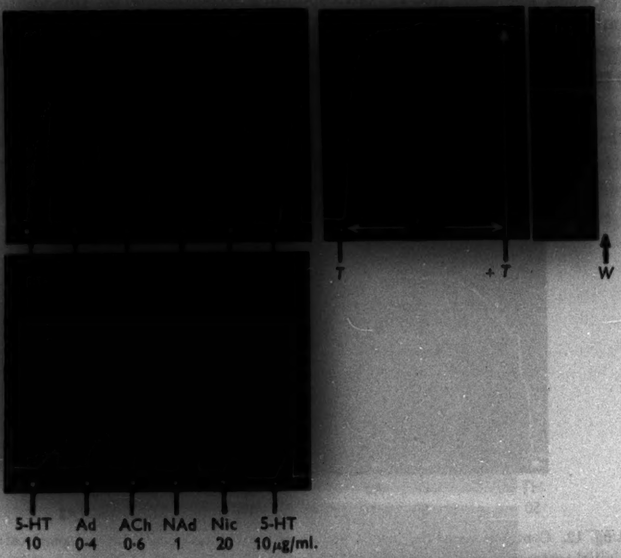
The 5-HT contractions are potentiated by cocaine (Text-fig. 6*b*), and antagonized by dibenamine (Text-fig. 7), dibenzyline (10^{-7}), atropine (Text-fig. 9), hyoscine (Text-fig. 10*b*) and dihydroergotamine. Although atropine, dibenamine, and dihydroergotamine antagonize 5-HT on various other smooth-muscle preparations (Gaddum & Hameed, 1954), these effects do not establish the presence of specific 5-HT receptors. Gaddum (1953) showed that the guinea-pig ileum was desensitized to 5-HT by saturating the receptors by adding excess tryptamine or 5-HT itself. The muscle then failed to respond to 5-HT whilst preserving its sensitivity to drugs which operated on receptors other than those for 5-HT. Corresponding experiments on the nictitating membrane gave a different result (Text-fig. 13). On the addition of either excess tryptamine or excess 5-HT the muscle failed to relax spontaneously and the responses to all other drugs became depressed.



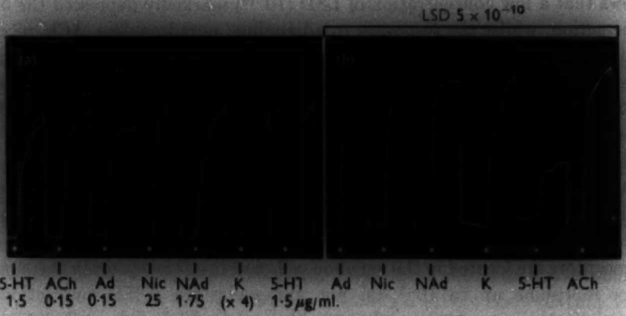
Text-fig. 12. Contractions of the medial muscle of the nictitating membrane (spinal cat). The initial contraction produced on the addition of 50 μg 5-HT to a 20 ml. bath was not increased when another 50 μg 5-HT was added. However, in the presence of 100 μg 5-HT additional contractions were produced by 10 and 100 μg adrenaline; a second addition of 100 μg adrenaline did not increase the contraction, which is therefore maximal.

On the other hand, lysergic acid diethylamide (LSD), a selective antagonist of 5-HT, also exerted this action on the nictitating membrane. Text-fig. 14 shows that a low concentration of LSD (10^{-10}) markedly depressed the 5-HT contraction, whilst the responses to adrenaline, noradrenaline, ACh, nicotine and potassium remained either unchanged or were even slightly increased. It was not possible to produce full antagonism because both LSD and its brom-derivative stimulate this muscle, as is shown in Text-fig. 14 by the rise of the base line. When the concentration of LSD was increased the muscle went into an irreversible spasm (see Text-fig. 8).

According to Gaddum & Picarelli (1957) the tryptamine receptors can be divided into the *M* receptors which are inactivated by morphine, and into the *D* receptors which are inactivated by dibenzyline. They conclude that the *M* receptors are located in the ganglion cells and the *D* receptors in the muscle. Since morphine up to 10^{-5} did not antagonize the 5-HT contractions of the nictitating membrane, whereas dibenzyline 10^{-7} did, and the same antagonistic action was found for dibenamine (Text-fig. 7), it would appear that the nictitating membrane possesses only the type *D* receptors located in the



Text-fig. 13. Contractions of the medial muscle of the nictitating membrane (chloralose), in *a* to 5-HT, adrenaline, ACh, noradrenaline and nicotine in the doses indicated. In *b*, tryptamine was added at *T* to produce a concentration of 100 μg/ml., whilst a further dose added 10 min later at $+T$ had no effect, showing that the contraction was maximal. At *c* the muscle failed to relax completely in the presence of the drug within the following period of 65 min, so drug washed out at *W*. At *d*, 45 min later, after the lever had returned to the original base line drugs given in *a* repeated.



Text-fig. 14. Contractions of the medial muscle of the nictitating membrane (spinal cat) to 5-HT, ACh, adrenaline, nicotine, noradrenaline and potassium in the doses shown; at *a* before, and at *b* after, the addition of LSD 5×10^{-10} to the bath.

muscle. Trendelenburg (1957) has shown that *in vivo* the 5-HT contractions of the nictitating membrane are not antagonized by morphine, thus supporting the findings *in vitro*.

Potassium. Contractions produced by potassium in smooth muscle preparations may be entirely direct effects on the muscle, or partly due to an action on nervous structures embedded in the preparation as in the guinea-pig and rabbit ileum, because in these preparations the potassium effect is to a varying degree reduced by hexamethonium (Feldberg, 1951). In the isolated nictitating membrane the potassium response was found to be insensitive to hexamethonium (Text-fig. 9), and is thus entirely a direct effect on the muscle. Increasing the potassium concentration in the Krebs's solution three- to fivefold has therefore frequently been used as a means of obtaining a directly elicited contraction when such a contraction was required for the analysis of responses to other drugs on the nictitating membrane.

Drugs to which the preparation is relatively or completely insensitive

Barium elicited a contraction of the muscle, but only in amounts of the order of 1 mg/ml. These amounts of barium caused irreversible damage to the muscle, as shown by the fact that subsequently it no longer responded to stimulant drugs.

Calcium in concentrations up to 1 mg/ml. was always inactive.

Histamine. Most preparations were insensitive to histamine even in large doses (100 μg/ml); but a few preparations responded to these doses with small contractions. Histamine never modified subsequent responses to other drugs.

Decamethonium and succinylcholine were inactive on the nictitating membrane *in vitro*. This is in agreement with the conclusions reached by Paton & Thompson (1953) as a result of their experiments on the membrane *in vivo*.

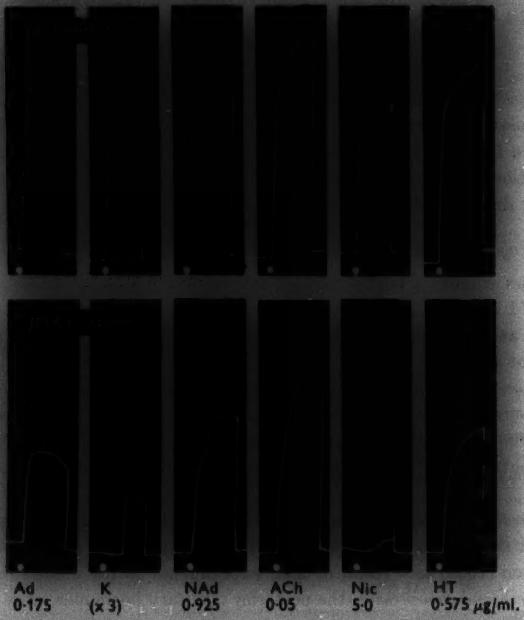
Oxytocin and vasopressin, the posterior pituitary hormones, were also found to be inactive. Oxytocin was tested in concentrations up to 0·2 u./ml. and vasopressin up to 0·3 u./ml.;

Bradykinin was another inactive polypeptide; it was tested in concentrations up to 2·5 μg/ml.

The effect of a general anaesthetic on the responses of the muscle

Isolated nictitating membranes obtained from cats anaesthetized with ethyl chloride, ether and intravenous chloralose (80 mg/kg) were found to be less sensitive to stimulant drugs than preparations obtained from cats anaesthetized with ether alone. The experiments made on the preparations from cats anaesthetized with ether alone had been carried out jointly with Dr I. R. Innes, of the Department of Physiology, University of Aberdeen. The question thus arose whether ether sensitized or chloralose depressed the muscle.

The following findings show that the long ether anaesthesia which preceded the isolation of the nictitating membrane did not sensitize the muscle. After allowing sufficient time for the ether to disappear, preparations made from spinal cats were as sensitive as those obtained from cats anaesthetized with ether throughout the whole dissection.



Text-fig. 15. Contractions of the medial muscle of the nictitating membrane (spinal cat) to adrenaline, potassium, noradrenaline, ACh, nicotine and 5-HT in the doses shown; at *a* before, and *b* after, the addition of chloralose 5×10^{-4} to the bath.

Since the preparations from spinal cats were more sensitive than those from chloralosed cats, chloralose seemed to exert a depressant effect and this could also be shown on the isolated muscle. Text-fig. 15 shows the depressant effects of chloralose 5×10^{-4} on the responses of the muscle to various drugs. Concentrations of chloralose from five to ten times less were still able to depress the contractions to some drugs, particularly nicotine. Assuming that chloralose is evenly distributed throughout the extracellular fluid of a cat receiving 80 mg/kg intravenously, a concentration of 5×10^{-4} could reasonably be present in the vicinity of the muscle. Thus, the finding that preparations obtained from chloralosed cats were less sensitive than preparations from spinal cats is not surprising, and for this reason all subsequent preparations have been obtained from spinal cats.

DISCUSSION

Although responses of the nictitating membrane of the cat have been analysed *in vivo* in many physiological and pharmacological experiments, some of the results have been controversial. It is universally accepted that this muscle responds to adrenaline and noradrenaline, and that it is more sensitive to adrenaline. Bacq & Fredericq in 1935 produced evidence that in some cats there is a cholinergic component in the post-ganglionic nerve supply to the nictitating membrane. This was confirmed by Burn & Trendelenburg (1954). The *in vivo* membrane was already known before 1935 to be sensitive to ACh and in fact Rosenblueth in 1932 showed that denervation of the membrane made the muscle hypersensitive to ACh as well as to adrenaline. Westcott & Christensen (1951) and Westcott *et al.* (1956) were the first to show that the ACh contraction of the isolated inferior muscle was potentiated by DFP and antagonized by atropine. However, Cervoni & West (1955) and Cervoni *et al.* (1956) found that not only the contractions induced by ACh, but also those induced by adrenaline and by preganglionic stimulation were potentiated by eserine and inhibited by atropine, and they therefore concluded that there were no specific receptors for ACh in the nictitating membrane. The results obtained with the preparation here described lead to a different conclusion. The effect of atropine obtained by Cervoni *et al.* (1956) is due to their use of a concentration far in excess of that required to antagonize equivalent doses of ACh, and this concentration may well have a non-specific depressant action which atropine is known to have in large doses (Gaddum, 1936). As has been shown in this paper, the high concentration of atropine they used will depress responses not only to adrenaline, but also to noradrenaline, nicotine and potassium, substances which are not specifically antagonized by atropine. The results of Cervoni *et al.* (1956) do not, therefore, invalidate the conclusion that there are specific receptors for ACh in the muscles of the nictitating membrane. Recently Feeney, Avery & Koppanyi (1956) and Feeney (1957) have done experiments on the nictitating membrane *in situ* which are in accord with these conclusions.

Contractions produced by 5-HT in both innervated and denervated membranes *in vivo* have been described previously (Reid & Rand, 1952; Erspamer, 1954; Lembeck, 1954; Lecomte, 1955; Trendelenburg, 1956), and 5-HT also acts on the membrane *in vitro*. The finding that it was not possible to obtain maximal contractions with 5-HT which were as large as those obtained with other stimulant drugs may be accounted for by the property of 5-HT to desensitize smooth muscle to its own stimulating effect. This desensitization was first observed by Gaddum (1953) for the 5-HT contractions of the guinea-pig ileum, and it also applies to the 5-HT contractions of the nictitating membrane. If this desensitization occurs quickly, as it apparently does, and is accentuated by increase of dosage, it is easy to understand that it occurs before the muscle has had time to contract fully, particularly since the contraction of the nictitating membrane is a relatively slow process. On the other hand, it is difficult to understand on these lines why the muscle failed to relax spontaneously if the 5-HT (and the same was found for tryptamine) was kept in the bath. In the guinea-pig's ileum spontaneous relaxation occurs without washing out the 5-HT or tryptamine. This inability of the nictitating membrane to relax in the continued presence of 5-HT or tryptamine made it difficult to use excess of these amines as a method for revealing the presence of tryptamine receptors in the muscle. Their presence, however, could be established by the use of LSD, since LSD is a powerful antagonist to 5-HT on this muscle. In this respect the nictitating membrane thus resembles the rat uterus and the vessels of the rabbit's ear, on which the effects of 5-HT are antagonized by small doses of LSD (Gaddum & Hameed, 1954) and differs from the guinea-pig ileum on which LSD has only a weak anti-5-HT action (Gaddum & Hameed, 1954). The tryptamine receptors of the nictitating membrane could be attributed as belonging to the type *D* receptors according to the classification of Gaddum & Picarelli (1957), since the action of 5-HT was not antagonized by morphine, but only by dibenzyline and also by dibenamine.

The responses produced by potassium assisted in locating the site of action of several drugs examined. Thus it could be shown that if atropine and hyoscine were given in concentrations higher than those required to block the ACh receptors they exerted a direct depressant action on the muscle, so that the responses of the muscle to potassium became reduced. The concentrations of atropine which depressed the muscle directly were, in fact, not much stronger than those required to antagonize the ACh responses. A similar condition pertains to the antagonistic action of atropine on the ACh and histamine contractions of the guinea-pig ileum (Feldberg, 1931). On the other hand, hyoscine antagonized the ACh responses of the nictitating membrane in much weaker concentrations than those which depressed the muscle, and is therefore a more specific antagonist to ACh on the nictitating membrane than atropine. Potassium also showed that dibenamine has a direct depressant action on the nictitating membrane in addition to its anti-adrenaline effect, since it depressed not only the responses to adrenaline and noradrenaline, but also to ACh and potassium. Dibenamine seems to have a similar directly depressant action on vascular smooth muscle, since Furchgott (1954) found that on this muscle it depressed not only the contractions to adrenaline and noradrenaline, but also those to ACh.

It is unlikely that the contractions of the isolated nictitating membrane produced by nicotine and tetramethylammonium are the result of ganglionic stimulation, because histological investigation failed to reveal ganglion cells in preparations which had responded to nicotine. Similar actions of nicotine have previously been described on the smooth muscle in the vessels of the frog's hind legs (Loewi, 1937), on the smooth muscle in the vessels of the rabbit's ear (Ginzel & Kottegoda, 1953) and in the walls of the bronchi (D. F. Hawkins & W. D. M. Paton, unpublished), structures which are normally assumed to be free from ganglion cells. It seems almost certain that the responses of the isolated nictitating membrane to nicotine and tetramethylammonium are produced indirectly through the release of adrenaline and/or noradrenaline. These responses were antagonized by hexamethonium, although histological investigation failed to reveal the presence of any ganglion cells by a standard silver staining method (Bielschowsky technique). Microscopical sections did, however, show large numbers of fine nerve fibres lying amongst the muscle and it seems most probable that these are the structures stimulated by nicotine and tetramethylammonium and are, in fact, the post-ganglionic nerve fibres to the muscle. These substances might stimulate either the trunk or the fine endings of these nerves and possibly produce a local axon reflex, as was suggested by Ginzel & Kottegoda (1953) when they investigated the action of nicotine in the vessels of the rabbit's ear. Although Evans & Schild (1953*a*, *b*) have demonstrated instances in which nicotine appears to act directly on smooth muscle, it is difficult to believe that this occurs in the normal isolated nictitating membrane. Future experiments on denervated membranes might clarify this problem. If, on the other hand, the observations of Evans & Schild (1953*a*, *b*) represent stimulation by nicotine of that uncertain histological entity known as the interstitial cell (Meyling, 1953), then stimulation of this histological entity may equally occur in the nictitating membrane.

The absence of any response of the isolated nictitating membrane to the end-plate depolarizing drugs decamethonium and succinylcholine confirmed the *in vivo* results of Paton & Thompson (1953). They found that both substances produced an apparent contraction of the membrane, which, however, was not a direct effect on smooth muscle, but resulted from activation of the extra-ocular muscles which are anatomically linked to the smooth muscle (Acheson, 1938). Histamine, to which so many smooth muscles are exquisitely sensitive, is remarkable for its lack of activity on this preparation. Although Rosenblueth (1932) and Gaddum & Goodwin (1947) stated that the intact membrane was activated by histamine, Burn & Trendelenburg (1954) suspected and subsequently proved that the effect obtained in the whole animal was not a direct one. It seems likely that any substance which can mobilize sympathins in the body may elicit similar responses.

SUMMARY

1. A method is described whereby an isolated preparation of the cat's nictitating membrane is obtained.

2. Most preparations showed no spontaneous activity. In some preparations in which spontaneous activity developed a brisk twitch could be induced by gentle stretching of the muscle.

3. Contractions of the isolated muscle were produced by adrenaline, noradrenaline, acetylcholine, acetyl-β-methylcholine, carbachol, pilocarpine, 5-hydroxytryptamine, potassium, barium, nicotine and tetramethylammonium.

4. *iso*Propylnoradrenaline in small doses caused the muscle to relax by an amount which was proportional to the degree of spontaneous contraction present. Large doses caused the muscle to contract, although sometimes both relaxation and contraction could be obtained successively with the same dose.

5. The muscle failed to respond to decamethonium and succinylcholine, posterior pituitary hormones (oxytocin and vasopressin), bradykinin or calcium. It was usually insensitive to histamine, except on a few occasions when large doses produced small contractions.

6. Pharmacological analysis showed that the muscle contained specific receptors for adrenaline and noradrenaline, for acetylcholine, and for 5-hydroxytryptamine.

7. The responses to nicotine and tetramethylammonium are explained by stimulation of fine post-ganglionic nerve fibres which lie amongst the smooth muscle and innervate it. Stimulation of these fibres leads to the release of adrenaline and/or noradrenaline which then cause contraction of the muscle.

8. When the influence of some general anaesthetics on the excitability of the muscle was examined, it was found that whilst chloralose depressed the muscle, ether had no effect.

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EXPLANATION OF PLATE

Photographs of the left eye of a spinal cat taken during a special dissection designed to illustrate the anatomical relationships between the visible nictitating membrane and the smooth muscle and other structures contained within it. This special dissection is not the same as that used before setting up the isolated preparation. The latter is described in Methods and differs from that shown here where, for example, the eyeball has been left *in situ*. During dissection the tissues were kept moist by means of a solution of warmed normal saline dripped on to them through a hypodermic needle, the end of which can be seen in Figs. 1 and 2.

Fig. 1. The nictitating membrane is lying freely half-way across the eye. A white silk ligature has been passed through the free edge of the cartilage.

Fig. 2. The membrane has been pulled completely across the eye by means of the ligature.

Fig. 3. Dissection of the nictitating membrane whilst held across the eye in the position seen in Fig. 2. The lids have been retracted and the lateral canthus incised in order to increase the exposure. The extent of the bellies of the medial (*m*) and inferior (*i*) smooth muscles is indicated by the black threads which pass behind each muscle. The harderian gland (*h*) is seen and the free edge of the cartilage is indicated by the white thread which passes through it. *b* = bony wall of orbit.

Fig. 4. Same dissection as in Fig. 3, with the membrane pulled away from the eyeball to show the angle at which the smooth muscle fibres are attached to the cartilage (compare with Text-fig. 1 *a*).

Fig. 5. The lids have been brought back into their original position as in Figs. 1 and 2 by removing the retractor and suturing the incision (*s*) made at the outer canthus. It is evident that the structures lying within the palpebral fissure consist almost entirely of cartilage and harderian gland (*h*); the smooth muscles lie beneath the lids.

(Facing p. 72)

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INTESTINAL TRANSFER OF SHORT-CHAIN FATTY ACIDS *IN VITRO*

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Interest in the absorption of short-chain fatty acids from the alimentary tract has been confined mainly to studies on ruminants. Very little information exists on absorption of short-chain fatty acids in other species. Höber (1945) included acetate in a list showing the relative rates of absorption of various anions from the intestine, but this was based on very early experiments (Höber, 1899) involving freezing-point measurements only. Deuel, Hallman & Reifman (1941) studied the absorption of short-chain fatty acids from rat intestine and gave the rates of absorption for all members of the series from acetic to tridecylic acid. No suggestion was made by these authors that any active process was involved in absorption of short-chain fatty acids, and Höber (1945), in considering the fatty acids along with other anions, thought that diffusibility was probably the predominant factor in determining the absorption rate. Recently Smyth & Taylor (1954, 1957*a*), using an *in vitro* intestinal preparation of rat intestine, found that the fluid transferred by the intestine contained short-chain fatty acids in a concentration higher than that in the lumen of the intestine, and in this respect fatty acids resembled glucose and L-amino acids, substances which there is reason to believe are transferred by an active process. One of the criteria of an active process is the ability to move substances against a concentration gradient; and it was therefore decided to test the capacity of the intestine to transfer short-chain fatty acids against a concentration gradient. A preliminary account of this work has been given by Smyth & Taylor (1957*b*).

METHODS

The intestinal preparation used was the sac of everted small intestine of the rat described by Wilson & Wiseman (1954). The procedure was, however, modified by using sacs of intestine 30 cm long instead of the very short sacs used by those authors. These sacs, prepared from the upper part of the small intestine, were suspended in 50 ml. bicarbonate saline (Krebs & Henseleit, 1932) and were shaken in conical flasks at a rate of 90 c/min. Except where otherwise stated, the bicarbonate saline contained 500 mg glucose/100 ml. The gas phase was 5 % carbon dioxide and 95 % oxygen except in the anaerobic experiments where 5 % carbon dioxide and 95 % nitrogen was used.

Each sac was filled with about 3 ml. bicarbonate saline which, relative to the size of the sac, is a much smaller volume than that used by Wilson & Wiseman (1954) and consequently produced no distension or turgidity. From some points of view distension may be advantageous, for example it may possibly improve the conditions for oxygen uptake by increasing the surface and diminishing the thickness of the intestinal mucosa. On the other hand, the increased pressure inside the sac will tend to oppose transfer of water from mucosal to serosal fluid, and we believe that the different degrees of distension of the sacs explain some of the differences between our results and those of Wilson & Wiseman (1954).

In describing the results the fluid inside the sac is referred to as the serosal fluid and the fluid in which the sac is suspended as the mucosal fluid. The piece of intestine used for the sac was weighed before (W_1) and after (W_2) filling with bicarbonate saline, and also at the end of the experiment (W_3). It was then opened and the serosal fluid drained out and the sac weighed again (W_4). The tissue was finally dried at 110° during the night and weighed (W_5). The initial serosal volume is obtained from $W_2 - W_1$ and the final volume from $W_3 - W_4$. The difference between initial and final volumes gives the volume of water transferred. (This cannot be directly determined from $W_3 - W_2$ because a certain amount of water is taken up by the tissue.)

The fatty acids were used as the sodium salts and were present initially in equal concentrations in the mucosal and serosal fluids. The final concentrations were determined by steam distillation in a Markham still of the filtrate obtained by deproteinizing with copper sulphate and sodium tungstate. For the subsequent titration 0·033 N-NaOH was used, with phenol red as indicator. From the concentrations of fatty acids and the initial and final volumes of the serosal solutions the initial and final amounts of fatty acid in the serosal fluid were calculated, and from these the amount of fatty acid transferred. A positive value for transfer means an increase in fatty acid on the serosal side and a negative value a decrease.

The units chosen for expressing the results deserve some consideration. In previous work on transfer and metabolism *in vitro* the Q notation has been frequently used. While this notation is well established in dealing with oxygen consumption, it has some serious disadvantages in expressing utilization and transfer, the chief of these being the use of the symbol μl. In the Q notation 1 μl. means $10^{-6} \times \frac{1}{22.4}$ mole irrespective of whether the substance is a solid, liquid or gas. In expressing transfer of fluids, however, the symbol μl. is now generally taken to mean 10^{-3} ml. We feel that two different uses of the symbol μl. can give rise to confusion, and we therefore propose to keep the symbol μl. (10^{-3} ml.) for volumes of fluid, and to express fatty acids as m-moles, μmoles or n-moles (10^{-9} mole). The concentration of fatty acid has been expressed as μmoles/ml. When the rate of transfer is expressed in μmoles/mg dry weight of tissue the number is inconveniently small, and it is therefore given as n-moles/mg dry weight of tissue/hr.

RESULTS

Movement of volatile fatty acids against a concentration gradient

In these experiments glucose was present initially in a concentration of 500 mg/100 ml. both in the mucosal and serosal fluid, and the fatty acid was also present initially in equal concentrations in the two fluids. The sacs were shaken under aerobic conditions for 90 min. The results are shown in Table 1. From this it is seen that there is a very considerable movement of fatty acid from the mucosal fluid to the serosal fluid. This results in the serosal fluid acquiring a higher concentration of fatty acid than the mucosal fluid, and hence the transfer of fatty acid must be against this concentration gradient. The movement of fatty acid is accompanied by movement of water, but it should be noted that the water movement is in the opposite direction to that which would be required to produce the observed differences in concentration of fatty acid in the mucosal and serosal fluids. Control experiments were carried out in which no added fatty acid was present, and in these cases no significant amounts of fatty acid were detected at the end of the experimental period.

Experiments have also been carried out with smaller concentrations of fatty acid than those given in Table 1. An 0·01 M solution is about the smallest concentration which, after deproteinization, gives a solution which can be conveniently estimated. Concentration against a gradient was observed with such solutions. This is of considerable interest as 0·01 M is in the range of concentrations of fatty acid found by Elsden, Hitchcock, Marshall & Phillipson (1946) to exist in the small intestine of the rat under physiological conditions.

TABLE 1. **Transfer of fatty acids against a concentration gradient by sacs of everted small intestine in aerobic conditions in the presence of glucose. Positive values for fatty acid and water transfer indicate movement to the serosal side**

	Fatty acid concentration			Rate of transfer of	
Fatty acid	Initial, mucosal and serosal (μmole/ml.)	Final, mucosal (μmole/ml.)	Final, serosal (μmole/ml.)	Fatty acid (n-mole/mg dry wt./hr)	Fluid (μl./mg dry wt./hr)
Acetic	22·6	20·6	28·6	278	8·54
	21·0	18·7	26·3	203	6·40
	20·4	18·1	29·3	228	6·25
	20·4	18·1	34·0	282	6·25
Propionic	19·7	18·4	29·9	237	5·82
	20·4	19·2	31·8	289	6·66
	20·9	19·1	33·7	273	5·89
	20·9	19·4	31·4	292	7·12
Butyric	18·4	15·6	35·8	314	6·01
	19·5	17·8	31·6	250	5·25
	18·6	15·2	33·2	321	7·07
	18·6	15·5	36·1	297	5·76

The figures for transfer expressed in n-moles may appear very small, and it is worth considering what contribution this amount of fatty acid could make to the total metabolic need. If the figures from the first line of Table 1 are taken, the transfer of fatty acid of 278 n-moles/mg dry weight/hr is equivalent to 5·62 mg/hr for 30 cm of intestine of a rat weighing 220 g. Since less than one third of the intestine has been used, and as the ileum may transfer acetate at a lower rate than the jejunum, the figure of 12 mg/hr might be taken for the whole intestine. The heat of combustion of acetic acid is 209 kcal/mole and therefore 12 mg acetic acid will yield 0·042 kcal. The rate of metabolism of the rat is given by Carr & Krantz (1942) as 0·183 g oxygen/100 g body weight, and a rat of 220 g will have a metabolic rate of 0·282 l. oxygen/hr, or about 1·4 kcal/hr. Thus, the acetate transferred could contribute about 3% of the total metabolic requirements. The amounts are thus not insignificant.

Under physiological conditions the mechanism would not have to transfer against a concentration gradient, and the amount moved could be considerably greater. Furthermore, as Elsden *et al.* (1946) have shown, the small intestine in the rat contains much less volatile fatty acid than the large intestine, and hence absorption from the small intestine may represent a relatively small part of the total volatile fatty acid used.

Effect of anaerobic conditions

The results of experiments carried out under anaerobic conditions are shown in Table 2. From this it is seen that in anaerobic conditions there is no transfer of fatty acid. The serosal concentration actually falls, and this could be due to diffusion of fatty acid into the tissue. Since the serosal volume is much smaller than the mucosal, only a small movement of fatty acid is required on the serosal side to produce a change in concentration. In anaerobic conditions the rate of water movement is greatly reduced but not completely abolished. This is in agreement with the results of Smyth & Taylor (1957 *a*) with a different type of *in vitro* preparation. Wilson & Wiseman (1954) found no transfer of water in everted sacs under anaerobic conditions, and usually even some reductions in volume of the sac. We believe the difference is due to the much greater distension of the sacs in their experiments, so that a considerable hydrostatic pressure existed to force water out of the sac.

TABLE 2. Transfer of fatty acids by sacs of everted small intestine in anaerobic conditions. Positive values for fatty acid and water transfer indicate movement to the serosal side

	Fatty acid concentration			Rate of transfer of	
Fatty acid	Initial, mucosal and serosal (μmole/ml.)	Final, mucosal (μmole/ml.)	Final, serosal (μmole/ml.)	Fatty acid (n-mole/mg) dry wt./hr)	Fluid (μl./mg dry wt./hr)
Acetic	22·3	22·4	20·5	− 3·2	0·40
	22·3	22·6	18·3	− 6·7	0·87
Propionic	19·4	19·2	14·2	−25·6	1·16
	19·4	18·9	11·9	−37·0	0·80
Butyric	18·3	17·7	12·5	−16·1	1·37
	18·3	18·7	13·5	−29·0	0·33

Effect of glucose on fatty acid transfer

Glucose has been shown by Fisher (1955) to be essential for water transfer in the intestine *in vitro*, and it was of interest to see whether it is necessary for fatty acid transfer. A series of experiments was therefore carried out, in conditions similar to those of the experiments in Table 1, except that glucose was absent from both the mucosal and serosal fluids. The results are shown in Table 3. It is evident that the absence of glucose caused reduction in transfer of both fatty acids and of water. It does not, however, abolish the transfer, and a considerable concentration gradient can still be built up. It will be noted that some water movement still takes place. Fisher (1955) and Smyth & Taylor (1957*a*) found an insignificant amount of water movement in the absence of glucose in *in vitro* intestinal preparations. We think this difference in water transfer is explained by the superiority of the everted sac of intestine over other *in vitro* preparations. In the everted sac conditions are such as greatly to favour the oxygenation of the intestinal mucosa and it is the general experience in our laboratory that the everted sac is more robust and has better powers of transfer of water and other materials than any other form of *in vitro* preparation. It seems likely that in these conditions the substrates present in the mucosa, without addition of glucose, are sufficient to maintain a small amount of water movement. Wilson (1956) has also recently obtained some water movement in sacs of everted intestine in the absence of glucose.

TABLE 3. Transfer of fatty acids by sacs of everted small intestine in aerobic conditions in the absence of glucose. Positive values for fatty acid and water transfer indicate movement to the serosal side

	Fatty acid concentration			Rate of transfer of	
Fatty acid	Initial, mucosal and serosal (μmole/ml.)	Final, mucosal (μmole/ml.)	Final, serosal (μmole/ml.)	Fatty acid (n-mole/mg dry wt./hr)	Fluid (μl./mg dry wt./hr)
Acetic	21·5	19·7	22·2	34·8	1·38
	21·5	19·5	22·3	46·0	1·85
	20·2	19·2	20·3	16·5	0·76
	20·2	18·8	24·0	58·1	1·38
Propionic	23·0	19·0	24·8	22·3	0·55
	23·0	19·8	23·0	45·6	2·07
	20·3	18·6	27·2	68·2	1·16
	20·3	19·4	25·7	78·1	1·72
Butyric	19·1	17·5	24·0	54·5	1·01
	19·1	17·7	23·8	80·4	2·02
	23·3	23·2	25·6	45·5	1·17
	23·3	22·6	26·0	25·9	0·25

Effect of inhibitors

The inhibitors tested were phlorrhizin and 2:4-dinitrophenol. The inhibitor was present initially in equal concentration in the mucosal and serosal fluids, and otherwise the experimental conditions were similar to those given in Table 1. The results are seen in Tables 4 and 5. The concentration of phlorrhizin used, 20 mg/100 ml., has been shown by Smyth & Taylor (1955*a*, *b*) to cause very considerable inhibition of transfer of both glucose and water in everted sacs of intestine, and it is found here to cause a similar inhibition of water transfer. While the transfer of fatty acid is also considerably reduced, it is seen that movement of fatty acid against a concentration gradient can still take place, and it is possible that the reduction in transfer of fatty acids is due at least partly to the reduction of water transport.

In the case of 2:4-dinitrophenol (10^{-42}M) there is also some reduction in water transfer, again in agreement with the results of Smyth & Taylor (1955*a*). In this case, however, while there is about the same degree of reduction in water transfer as occurs with phlorrhizin there is a much greater reduction in transfer of fatty acids, and in no case is there any evidence of movement against a concentration gradient. It would thus seem that 2:4-dinitrophenol has a definite effect on fatty acid movement apart from its effect on water transport.

TABLE 4. Effect of phlorrhizin on fatty acid transfer by the intestine under aerobic conditions in the presence of glucose. The phlorrhizin was present in all cases in an initial concentration of 20 mg/100 ml. Positive values for fatty acid and water transfer indicate movement to the serosal side

	Fatty acid concentration			Rate of transfer of	
Fatty acid	Initial, mucosal and serosal (μmole/ml.)	Final, mucosal (μmole/ml.)	Final, serosal (μmole/ml.)	Fatty acid (n-mole/mg dry wt./hr	Fluid (μl./mg dry wt./hr)
Acetic	20·3	19·5	24·3	75	2·08
	20·6	19·2	25·6	141	4·00
Propionic	20·4	19·1	34·2	171	2·12
	16·1	14·7	24·2	146	3·35
Butyric	18·4	16·7	30·6	128	1·97
	20·0	19·0	26·8	89	1·34

TABLE 5. Effect of 2:4-dinitrophenol on the transfer of fatty acids by the intestine under aerobic conditions in the presence of glucose. The inhibitor was present in all cases in a concentration of 10^{-4}M. Positive values of fatty acid and water transfer indicate movement to the serosal side

	Fatty acid concentration			Rate of transfer of	
Fatty acid	Initial, mucosal and serosal (μmole/ml.)	Final, mucosal (μmole/ml.)	Final, serosal (μmole/ml.)	Fatty acid (n-mole/mg dry wt./hr)	Fluid (μl./mg dry wt./hr)
Acetic	20·0	20·0	16·8	12·5	2·09
	20·3	20·4	20·5	25·0	1·17
Propionic	19·6	19·2	17·3	8·5	1·31
	16·1	15·8	13·1	19·6	3·03
Butyric	19·5	19·2	17·4	16·5	1·73
	20·0	19·8	17·3	41·1	3·64

DISCUSSION

The experiments described show that acetic, butyric and propionic acids are transferred by rat intestine *in vitro* against a concentration gradient. This transfer against a concentration gradient is completely abolished by anaerobic conditions and by the presence of 2:4-dinitrophenol. It is also greatly reduced by the absence of glucose and by the presence of phlorrhizin. These results suggest that the movement of the lower fatty acids depends on factors other than diffusion, and also that it is related to the metabolic activity of the intestine. The transfer could therefore be said to involve an active process.

Whether this is an active process specifically related to fatty acid transfer is much less certain. Smyth & Taylor (1957*a*) have discussed a possible mechanism which could account for accelerated transfer of fatty acids by the intestine, and even transfer against a gradient, without requiring a specific active transfer of fatty acids. Sodium is present in the fluid transferred through the intestinal wall in the same concentration as in the mucosal fluid. It is possible that the movement of anions is linked to movement of sodium, and there may be competition between the various anions present in the intestinal lumen. For some reason, possibly related to their greater lipoid solubility, fatty acids may pass through the intestinal wall more readily than chloride, and such a process could result in building up a concentration gradient of fatty acids against which the movement takes place. This view is consistent with the findings of Wilson (1954) and of Smyth & Taylor (1957*a*) that chloride moves through the intestine at a slower rate than sodium. In the case of some non-electrolytes Höber (1945) has concluded that lipoid solubility may be an important factor in absorption rate, and a similar principle could apply to electrolytes also. It is thus possible to conceive of an active process which might be responsible for fatty acid transfer, but which might not be specifically related to fatty acids.

On the other hand, a consideration of the effect of the inhibitors suggests that fatty acid transfer cannot be entirely explained as a result of sodium or water transfer. The effects of the two inhibitors used differ in some striking ways. Thus while phlorrhizin in the concentration used inhibited both fatty acid transfer and water transfer to a considerable extent, it did not abolish the establishment of a higher concentration of fatty acid on the serosal side than on the mucosal side. For these reasons we might think that the inhibiting effect of phlorrhizin on fatty acid transfer is not a specific one, but is possibly due to reduction of fatty acid movement secondary to the reduction of the water or sodium movement. On the other hand, dinitrophenol exerted a greater inhibiting action on fatty acid transfer than on water transfer, and also abolished completely the establishment of a concentration gradient. Since the two inhibitors had about the same effect on water transfer, this would suggest that there is some process related to fatty acid transfer affected particularly by dinitrophenol. There is clearly a resemblance between fatty acid transfer and amino acid transfer, as Friedlander & Quastel (1955) have shown that the transfer of some L-amino acids *in vitro* is markedly inhibited by dinitrophenol but only slightly by phlorrhizin. It seems safe to conclude that the transfer of fatty acids by the intestine is brought about by processes dependent on the metabolic activities of the intestine and that these processes are not identical with, although they may be related to, those concerned with the transfer of water and sodium.

Elsden *et al.* (1946) found from 0·04 to 0·10% acetic acid in the small intestine of the rat. The concentrations we have used are of the same order of magnitude, and it is therefore likely that the transfer mechanism we have described plays a physiological role in absorption of short-chain fatty acids.

SUMMARY

1. Experiments are described in which transfer of short-chain fatty acids by an *in vitro* intestinal preparation is described.

2. The transfer takes place against a concentration gradient, is abolished by anaerobic conditions and reduced by absence of glucose. The mechanism is also affected by phlorrhizin and by dinitrophenol.

3. Possible explanations for the transfer of fatty acids against a concentration gradient are discussed.

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THE RESPONSE OF THE NEW-BORN PUPPY TO WATER, SALT AND FOOD

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It has been shown by McCance & Widdowson (1956) that growth is one of the most important mechanisms in maintaining the composition of the body fluids of the new-born piglet within normal limits. The authors were concerned at that time chiefly with the concentration of urea. They subsequently showed (McCance & Widdowson, 1957) that when increasing amounts of NaCl were added to the food of piglets and babies there was a progressive expansion of the extracellular fluids and a rise in their osmotic pressure. This effect was demonstrated even more clearly in piglets when the milk and salted milk were replaced by water and salted water. In parallel with the work on babies and piglets experiments have been made on new-born puppies, but this species has reacted in some ways so differently from the other two that it seems best to present the results in a separate paper.

METHODS

The puppies were pure-bred bull terriers or the result of a mongrel × pure bull terrier cross. Each puppy was removed from the mother as soon as it was born, its bladder emptied as described by McCance & Widdowson (1955), and the urine discarded. When five of about the same size had been born and had had time to dry, they were weighed and placed in separate compartments of a long wooden box, each puppy lying on a piece of plastic material. The box was kept in a warm place at a temperature of about 21° C. The first puppy was given water, the second salted water, the third an amount of milk reckoned to contain as much water as each of the first two puppies had been given, and the fourth the same volume of salted milk. These liquids were administered two-hourly by stomach tube for the duration of the experiment, which was 24 hr. The fifth puppy was given nothing. In the first experiment dog's milk collected from another bitch and stored at −15° C was given. In the other experiments sow's milk was used. This overcame the labour of collecting sufficient dog's milk, and the puppies increased satisfactorily in weight on it. The sow's milk contained considerably less Na and Cl and somewhat less K than the dog's milk (Table 1), and in order to make its composition more like dog's milk appropriate amounts of NaCl and KCl were added to all the sow's milk. The 'salted milk' was further treated with larger amounts of NaCl.

The quantities of water and milk to be given were calculated on the basis of body weight so that they were similar to those used in the experiments on piglets. The concentration of salt in the salted water, and in the water of the salted milk, was 0·7 %. This was the mean concentration used for piglets, and the one used in the last two experiments on babies (McCance & Widdowson, 1957). The three species, therefore, have each been subjected to a similar stress.

Urine was collected two-hourly or oftener while the experiment lasted and stored under toluol. The amounts passed in successive periods of 8 hr were analysed separately. The puppies were not bled at birth, but some blood was taken from the mother just before delivery for the determination of urea. At the end of 24 hr, 5–10 ml. of blood was taken from the heart of each puppy, the animal was killed by injecting air into its heart, weighed and kept in a refrigerator at 4° C until the dissection could be carried out. Further details about the techniques and the chemical and physical methods employed may be found in the papers to which reference has already been made.

Four litters of puppies have been used for this work but the first was expended on an incomplete pilot experiment, and the results for this are not being presented. The other three litters have been handled as described, and the figures given in the tables are the means of these three experiments.

TABLE 1. Composition of dog's milk and sow's milk

		Sow's milk†	
	Dog's milk*	Average	Range
N (g/100 g)	1·15	0·78	0·70–0·85
Na (m-equiv/l.)	28	11	8–13
K (m-equiv/l.)	39	28	25–33
Cl (m-equiv/l.)	57	17	15–19

* Pooled samples from 3 bitches. † Samples from 8 sows.

RESULTS

Table 2 shows the effect of giving water and milk and of adding salt to these fluids on the visible water balance, the Na and Cl balances, the serum Na, Cl and total osmolar concentration, and the body weight. Results for the animals given nothing at all are also included.

When water alone or salted water was given the volumes of urine were large but there were positive water balances amounting to about 100 ml./kg/24 hr. Only the puppies given nothing had negative water balances and they, therefore, were the only ones in a state of hydropenia at death (see Table 3). The puppies which were given milk had a considerable gain of water which was associated with a retention and utilization of N and K, and was due largely to physiological growth (see Table 4). When the milk was salted the positive water balance was also large, and the urine volumes of the groups which were fed were less than half of those given water or salted water. The Na and Cl balances may be described together. There were negative balances of more than 9 m-equiv/kg/24 hr when water was given alone and positive balances of about 7 m-equiv/kg/24 hr when the animals received the same volume of 0·7 % saline. There were positive balances on milk alone which were of course due to the requirements for growth. When the milk was salted larger amounts of Na and Cl were excreted, but more was retained than the requirements for growth demanded, or than the puppies on salted water retained. This may have been due to their smaller urine volumes. The puppies given nothing excreted between 4 and 5 m-equiv/kg/24 hr.

The puppies given water alone gained weight. The water which they did not excrete (see above) diluted their body fluids, as is shown by the low figures for Na, Cl and total osmolar concentration in their sera. The puppies given 0·7% saline gained a little weight, and the osmotic pressure within their bodies was just about maintained, as is shown by the serum findings (also given in Table 2). The puppies given milk gained most weight and grew well. There is nothing to indicate that they were in any way abnormal. The ones given salted milk also grew well as shown by the N and K balances, but they gained less weight than those given unsalted milk and retained more salt than their requirements. Their serum Na, Cl and total osmolar concentrations rose to the levels found in the puppies given nothing, and these puppies were naturally dehydrated.

TABLE 2. The effects of adding salt to the water and milk administered to puppies on the visible water balance, the Na and Cl balances, the concentration of Na and Cl in the serum and its total osmolar concentration, and on the body weight

Fluid administered	Water	Salted water	Milk	Salted milk	Nothing
Water intake (ml./kg birth wt./24 hr)	256	253	256	255	0
Urine volume (ml./kg birth wt./24 hr)	135	158	37	59	31
Visible water balance (ml./kg birth wt./24 hr)	+121	+95	+219	+196	−31
Na intake (m-equiv/kg birth wt./24 hr)	0	27·2	9·4	27·7	0
Na excretion (m-equiv/kg birth wt./24 hr)	9·2	21·2	2·5	7·9	4·4
Na balance (m-equiv/kg birth wt./24 hr)	−9·2	+6·0	+6·9	+19·8	−4·4
Cl intake (m-equiv/kg birth wt./24 hr)	0	27·2	14·3	31·2	0
Cl excretion (m-equiv/kg birth wt./24 hr)	9·5	19·0	5·1	12·2	4·9
Cl balance (m-equiv/kg birth wt./24 hr)	−9·5	+8·2	+9·2	+19·0	−4·9
Na in serum (m-equiv/l.)	120	144	141	158	157
Cl in serum (m-equiv/l.)	77	99	93	107	105
Total osmolar concn. (m-equiv/l.)	243	285	301	306	308
Change in body weight (g/kg birth wt./24 hr)	+69	+3	+178	+139	−93

TABLE 3. The effect of adding salt to the water and milk administered to puppies on the percentage of water in the carcasses, livers, hearts and kidneys

(g water/100 g fresh tissue)

Fluid administered	Water	Salted water	Milk	Salted milk	Nothing
Whole carcass	84·3	82·9	82·6	82·2	80·8
Liver	81·5	81·6	79·5	78·7	78·9
Heart	84·7	83·8	82·7	83·0	82·5
Kidneys	87·6	86·9	85·9	85·3	86·0

Table 3 gives the percentage of water found in the carcasses, liver, heart and kidneys of the five groups of puppies. The findings for the carcasses agree with those to be expected from the results given in Table 2. The percentage of water in the puppies given water was abnormally high. The percentage in the ones given salted water was about the same as in those given milk. The percentage in the ones given salted milk was also the same, but the composition of the extracellular fluids was not, for they had about the same concentrations of Na, Cl and total osmolar material in them as those of the puppies given nothing, and these were abnormally high owing to dehydration.

Table 4 shows the N balances, the K balances, the serum urea, N appearing as end products and the haematocrit levels. The nitrogen appearing as end products of protein metabolism was calculated from the sum of the increased amount of nitrogen as urea in the body water and the total nitrogen excreted in the urine.

TABLE 4. The effects of adding salt to the water and milk administered to puppies on their nitrogen and potassium balances, the concentration of urea in the serum, the amount of nitrogen appearing as end products of protein metabolism and on the haematocrit level

Fluid administered	Water	Salted water	Milk	Salted milk	Nothing
N intake (mg/kg/24 hr)	0	0	2500	2320	0
N excretion (mg/kg/24 hr)	194	182	246	336	162
N balance (mg/kg/24 hr)	−194	−182	+2254	+1984	−162
K intake (m-equiv/kg/24 hr)	0	0	11·3	11·1	0
K excretion (m-equiv/kg/24 hr)	4·0	4·9	2·8	4·0	5·1
K balance (m-equiv/kg/24 hr)	−4·0	−4·9	+8·5	+7·1	−5·1
Serum urea (mg/100 ml.)	38·7	30·2	86·1	63·7	85·3
N appearing as end products (mg/kg/24 hr)	229	184	514	490	358
Haematocrit (%)	64·1	57·3	52·6	51·2	57·9

The puppies given water had slightly larger negative N balances than those given 0·7 % saline, but the results were of the same order and both negative balances were greater than those for the puppies given nothing. The puppies fed on milk and salted milk had very large positive N balances and retained respectively 90 % and 86 % of the protein N in the food. The K balances moved with the N balances, but the losses of K exceeded those to be expected from the losses of N when the puppies received no food. The concentration of urea in the serum at birth has been taken to be 25·2 mg/100 ml., which was the average value found for the three mothers. It rose in all five groups of puppies, most in those given milk or nothing and least in those given 0·7 % saline. The quantity of N appearing as end products of metabolism was about 200 mg/kg/24 hr when the puppies were given water and 0·7 % saline, and about 500 mg/kg/24 hr when puppies were fed. The figure was between the two when the puppies were given nothing.

The haematocrit values were highest on water alone, lower and similar on 0·7 % saline and on nothing at all, and lower still on milk and salted milk.

DISCUSSION

The metabolism of water and salt

It is impossible to offer a complete explanation for all the results in Tables 2–4, but the main ones are clear, and from the work on the three litters certain facts stand out for discussion. If the findings for the animals which were given water are considered first, it is evident that when the puppies were given about 250 ml. of water/kg/24 hr (*a*) they did not excrete it all, (*b*) they excreted much of the Na and Cl in their extracellular fluids. In both respects they differed from new-born piglets, for reference to the figures given in previous papers shows that these animals excreted a volume of water equal to that administered, and only a trifling amount of Na and Cl. When the water was replaced by an equal volume of 0·7 % saline, puppies excreted as much or more water and about 75 % of the Na and Cl. Piglets excreted little of the water and practically none of the Na and Cl. When the puppies were given nothing at all they excreted about 4 m-equiv/kg/24 hr of Na and Cl, whereas piglets excreted less than 1 m-equiv. Thus when both species are unfed, new-born puppies differ from new-born piglets in that: (*a*) they excrete water less freely when it is given without salt; (*b*) they excrete Na and Cl much more freely, so much so that their urine always contains appreciable amounts of salt and they are depleted of salt by a diuresis (in this connexion it is perhaps worth referring to the fact that dog's milk contains more than twice as much Na and Cl as sow's milk); (*c*) owing to characteristic (*a*), puppies become hypotonic after the administration of 250 ml./kg/24 hr of water, whereas piglets do not, and owing to characteristic (*b*) puppies do not suffer from hypertonic expansion of their extracellular fluids when they are given 250 ml./kg/24 hr of 0·7 % saline, whereas piglets do.

When the puppies were given milk, whether salted or not, they grew rapidly, as did piglets, but requirements of water and salt for growth and the effects of growth complicate the interpretation of the findings in both species, though the main results are clear. When the milk was salted puppies excreted more water and more salt than they did from milk alone, whereas piglets excreted much less water and little extra salt. The puppies, however, did not excrete all the extra salt they had been given, and since most of the water retained by the puppies was probably used for growth, whatever the kind of milk, the effect of salt on the animals was to leave their body fluids normal in volume but hypertonic. The effect on the piglets was to produce hypertonic expansion of the extracellular fluids. Since human infants have been shown to behave very like piglets when given an excess of salt, puppies differ from both species in some rather arresting ways. The differences are, however, quantitative rather than absolute, for puppies show the usual infantile retentions of Na and Cl when compared with adult dogs. The incomplete excretion of water has been demonstrated before (McCance & Widdowson, 1955). It is not peculiar to puppies (Lasch, 1923; Heller, 1951; Ames, 1953; McCance, Naylor & Widdowson, 1954), and it was shown by McCance & Widdowson (1955) that even at the height of the diuresis the urines of puppies contained Na and Cl, and were less hypotonic than those of full grown dogs. The present results, therefore, confirm these findings in puppies.

Puppies may be considered on various criteria, such as their closed eyes and general development, to be less mature than piglets at birth, yet in the freedom with which they excrete salt they appear to be more mature. The matter is complicated and requires more investigation, for the activity of the suprarenal hormones is particularly obscure at this age. General development, however, should evidently not be used without due consideration as an index of specific functional maturity.

Food, growth and homoeostasis

The haematocrits. The order in which the haematocrit levels appear are reasonable and understandable in a general way. The low levels in the puppies which were fed were not due to anaemia, but to the protein in the food leading to the synthesis of plasma protein and its appearance in the blood vessels. The findings in piglets were similar (McCance & Widdowson, 1957), modified to some extent by the differences in the rates at which water and salt were excreted by the two species and which have already been discussed.

Protein catabolism. It was shown in piglets (McCance & Widdowson, 1957) that expanding the extracellular fluids by adding NaCl to the food or water reduced the break-down of tissue protein. An effect of the same magnitude could not be expected in puppies since the addition of salt did not lead to the same expansion of the extracellular fluids. There are, nevertheless, signs of the same phenomenon (see Table 4), and it is worth noting that the animals given either water or salted water had a lower tissue break-down (see p. 6) than those which were given nothing at all and were in consequence dehydrated. Hydropenia, whether due to water deficiency or to salt deficiency, has previously been shown to increase tissue protein catabolism (Leaf & Couter 1949).

Serum urea. The importance of growth as an element in homoeostasis in the new-born has previously been pointed out (McCance & Widdowson, 1956, 1957). Puppies provide another good example of this, for reference to Table 4 shows that the serum urea was highest in the animals which were fed, and reached a level of 86·1 mg/100 ml. in those which were given milk. This figure is not unduly high for a well-fed new-born puppy, but high enough above the level at birth to show that the renal excretion of urea had not kept pace with its formation. Yet these animals had utilized 2254 mg of their food N/kg body wt./24 hr for purposes of growth, which was 90% of the total amount. Their kidneys could only have excreted a fraction of this amount.

SUMMARY

1. Unfed new-born puppies were given water, 0·7% saline, dog's or sow's milk, salted milk or nothing at all for the first twenty-four hours of their lives.

2. The water was incompletely excreted, but that which was excreted was accompanied by loss of salt originally in the body.

3. Administered salt was tolerably well excreted and did not lead to hypertonic expansion of the extracellular fluids, as it has been shown to do in piglets and human infants.

4. The puppies that received milk grew well.

5. Growth was a most important element in the maintenance of homoeostasis.

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THE RESPONSE OF THE NEW-BORN PIGLET TO AN EXCESS OF POTASSIUM

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In experiments on the nitrogen metabolism of piglets during the first days of life (McCance & Widdowson, 1956) some of the piglets were given an evaporated cow's milk mixture. This contained more sodium, chloride and potassium than sow's milk, and piglets having it developed hypertonic expansion of the extracellular fluids. This was more fully investigated by McCance & Widdowson (1957), and it was then shown that human infants reacted in a very similar way. The animals in the first experiments also retained more potassium from the cow's milk mixture than they appeared to require for co-ordinated growth. The interest of this is not confined to piglets, for something similar has been shown to take place in human infants (M. O. Beem & C. A. Smith, personal communication). Further experiments therefore were designed to study this retention of potassium under more controlled conditions than were possible in 1956. The experiments were planned on the lines of those carried out on sodium chloride loading and are reported in this paper.

METHODS

The general arrangements were the same as those described by McCance & Widdowson (1956, 1957) and only special points need be described in detail. Three litters have been used. Each has been treated in an almost identical manner and all have given similar results. The first piglet in each litter was given water, and the second an equal volume of water to which KCl (120 m-equiv/l.) had been added. This contained the same concentration of chloride as the 0·7% NaCl solution used in the previous experiments (McCance & Widdowson, 1957). The third piglet was given a volume of sow's milk $\frac{5}{4}$ times as great as the volume of water. This made the intakes of water about equal since the sow's milk used contained 15–20% of solids. The fourth piglet was given a volume of sow's milk equal to that given to the third, but KCl was added to make the concentration of potassium in the water of the milk equal to the concentration of potassium in the water given to the second piglet. This made the potassium intake of piglets two and four about the same and roughly three times that of the third piglet, which was having sow's milk. These piglets were fed two-hourly as before. The fifth piglet was given nothing.

The experiments, like the ones on sodium chloride, lasted for 40 hr and the urines were again collected in three periods of 12, 12 and 16 hr and analysed separately. The reducing sugar in the serum was determined by the method of Nelson (1944), and the other chemical methods have all been described before. The figures given in the tables and text are the averages of the three experiments unless it is made clear that individual results are being considered.

RESULTS

Matters of observation

At the environmental temperatures of 23–26° C at which these experiments were carried out the new-born pig appears to stand up to starvation well, and the ones given water but no food were lively and fit at the end of the 40 hr. The ones given the solution of potassium chloride in water were not so lively at the end. The hind quarters of one were paralysed for the last few hours of its life, and all four limbs of another—the cry of this animal was also weak. No other effects of raising the potassium intakes were observed visually, and in particular there was none of the pinkness and oedema so obvious when the animals were loaded with sodium chloride (McCance & Widdowson, 1957).

TABLE 1. The effects of administering KCl to piglets on the body weight, on the concentrations of potassium, sodium, chloride, urea and creatinine in the serum and on the blood sugar and haematocrit levels

Fluid administered	Water	Water + KCl	Milk	Milk + KCl	Nothing
Change in body weight (g/kg birth weight)	−111	−104	+109	+103	−111
Serum urea (mg/100 ml.)	41·8	29·3	19·8	19·2	63·5
Serum creatinine (mg/100 ml.)	1·47	1·43	1·33	1·22	1·55
Serum K (m-equiv/l.)	4·3	9·3	4·3	5·9	5·2
Serum Na (m-equiv/l.)	138	134	141	146	140
Serum Cl (m-equiv/l.)	101	106	106	109	107
Blood sugar (mg/100 ml.)	70	206	140	155	90
Haematocrit (%)	45·9	53·1	38·9	38·3	41·2

Quantitative measurements

Table 1 shows the average changes in the body weights, and the concentrations of urea, creatinine, potassium, sodium and chloride in the serum at death, the blood sugar concentrations and the haematocrits. The animals given water and the potassium solution lost about the same amount of weight, and the animals getting milk and 'potassium' milk gained weight satisfactorily and to a similar extent. Thus loading with KCl had no effect on the body weight, whether the animals were given water or milk, and this is to be contrasted with the gain of weight and extensive oedema which was induced by loading with NaCl. The addition of KCl to the water raised the serum K from 4·3 to 9·3 m-equiv/l., and its addition to the milk from 4·3 to 5·9, a much smaller increase and one not much greater than that caused by the complete deprivation of all food and water.

The serum urea was lowest in the two groups of animals fed on milk. This has been the usual experience when the piglets have been hand fed. The rise was greatest when the animals were given nothing. The concentrations of creatinine paralleled those of urea. The blood sugars were within the normal fasting limits for adult animals in the piglets given water or in those given nothing and this has always been so at the high room temperature at which these experiments have been made (Goodwin, 1957). The blood sugar levels were much higher in the animals given the solution of potassium in water and the height seemed to vary with the extent of the paralysis. The blood sugar levels of the animals given milk were above those of the fasting animals and the addition of potassium to the milk did not make them significantly higher. The concentration of sodium in the serum was not consistently altered by the addition of KCl to the water or to the milk, but that of the chlorides was slightly raised. Laragh & Capeci (1955) found that heavy loading with potassium raised the serum sodium in dogs. The haematocrit values were lowest when the animals were fed, and higher and about the same when the animals were given nothing or only water. These have been the usual findings in piglets. The piglets given the KCl in water had the highest haematocrit values in all three experiments, and this is to be contrasted with the results which followed the administration of NaCl.

TABLE 2. The effects of adding KCl to the water and milk on the volumes of urine, the visible water balance and on the Na, K, Cl and N balances

(All results expressed per kg birth weight per 24 hr)

Fluid administered		Water	Water + KCl	Milk	Milk + KCl	Nothing
Water	Intake (ml.)	220	210	246	238	0
	Urine vol. (ml.)	234	216	169	145	30
	Visible water balance (ml.)	−14	−6	+77	+93	−30
K	Intake (m-equiv)	0	25·5	8·1	26·7	0
	Excretion (m-equiv)	3·9	22·6	1·7	16·2	3·6
	Balance (m-equiv)	−3·9	+2·9	+6·4	+10·5	−3·6
Cl	Intake (m-equiv)	0	25·7	5·3	22·5	0
	Excretion (m-equiv)	2·2	23·8	2·8	17·0	1·1
	Balance (m-equiv)	−2·2	+1·9	+2·5	+5·5	−1·1
Na	Intake (m-equiv)	0	0	3·04	3·42	0
	Excretion (m-equiv)	0·15	4·17	0·23	0·42	0·06
	Balance (m-equiv)	−0·15	−4·17	+2·81	+3·00	−0·06
N	Intake (mg)	0	0	2250	2238	0
	Excretion (mg)	214	227	270	225	143
	Balance (mg)	−214	−227	+1980	+2013	−143
	Appearing as end products (mg)	238	226	254	209	223

Table 2 shows the visible water balances, and the potassium, sodium, chloride and nitrogen balances of the five groups of animals. The piglets given water and potassium solutions had slightly negative water balances, and the animals given nothing somewhat larger ones. The piglets which were fed had large positive water balances and loading with KCl made little difference to them. These visible water balances are in general agreement with the changes in body weight shown in Table 1, but the effects of KCl are unlike those of NaCl, which greatly increased the retention of water. The piglets given water alone lost 3·9 m-equiv K/kg starting weight and those given the potassium solutions gained 2·9 m-equiv. Thus the bodies of animals given the KCl retained about 7 m-equiv more K/kg/24 hr than those of the animals given water alone. The piglets given milk grew well and this led to the retention of 6·4 m-equiv K/kg/24 hr. The animals getting the 'potassium' milk had, however, larger positive balances, amounting to 10·5 m-equiv/kg/24 hr, so that at death they had retained about 4 m-equiv more K/kg/24 hr than their littermates given normal milk. The animals given nothing lost 3·6 m-equiv K/kg/24 hr, which agrees very well with the losses sustained by the animals having water.

The potassium was given as the chloride so that the animals getting the KCl had large intakes of chlorides. These chlorides were, however, more completely excreted than the potassium, and the large retentions which followed the administration of NaCl in the previous experiments must be attributed entirely to the retention of the sodium ion. This appears also to be so in human cardiac failure (Friedberg, 1957). The piglets getting water alone had very small negative sodium balances. This is usual in piglets and agrees with the findings in the animals given nothing at all. The negative balances were materially increased by the addition of potassium chloride to the water in all three experiments. Laragh & Capeci (1955) did not find this in adult dogs. The piglets getting milk had small positive sodium balances associated with true growth, and this was not affected by the addition of potassium to the food.

The negative nitrogen balances were least in the animals given nothing at all. This result has been obtained and discussed before. The negative balances on water and on potassium solutions were of the same order and so were the amounts of nitrogen appearing as end products of metabolism. There was no valid evidence of the nitrogen-sparing effect which followed the administration of NaCl. The animals given milk and 'potassium' milk both had large positive balances due to growth, and incorporated the usual 85–90% of their ingested protein N into their tissues. The nitrogen appearing in the form of end products (calculated from the change in serum urea and the total nitrogen excreted) was less when the animals were given 'potassium' milk than when they were given normal milk. This was largely due to one animal which gave a surprisingly low result and the difference shown in the table may not be a real one.

Table 3 shows the composition of the skeletal muscles, liver and heart, and the weights of the two last as percentages of the body weight at death. Loading with KCl made no difference to the percentage of water in the tissues at death, and analysis of the carcasses confirmed this. Loading with NaCl always raised the percentage (McCance & Widdowson, 1957). The reduced amounts of water in the livers of the animals which were fed is a confirmation of previous results.

The administration of the potassium chloride led to an increase in the potassium in skeletal muscle from 75·4 to 91·7 m-equiv/kg when the animals were not fed, and from 69·2 to 74·0 when the animals were fed. There was no corresponding increase in the percentage of nitrogen, and consequently a fall in the N:K ratio in both groups of animals given potassium. In the liver and heart the effects of giving potassium were smaller and somewhat equivocal. This was not unexpected in the light of work which has been carried out on potassium deficiency (Gardner, Talbot, Cook, Berman & Uribe, 1950). Its administration made no difference to the weight of the liver. The increased size of the organ in the animals which were fed—both absolutely and as a percentage of the body weight—has been observed and reported before (McCance & Widdowson, 1956). The hearts of the animals which were given potassium were somewhat smaller than their controls but the differences may have been due to chance. The concentration of potassium in the organ was not consistently raised by potassium administration although it would appear to have been so when the animals did not get milk.

TABLE 3. The effect of administering KCl to piglets on the concentration of K, Na, Cl and N in the tissues

Fluid administered	Water	Water + KCl	Milk	Milk + KCl	Nothing
Muscle					
Water (g/100 g)	85·1	84·5	84·0	84·0	84·9
K (m-equiv/kg)	75·4	91·7	69·2	74·0	75·5
Na (m-equiv/kg)	39·0	30·7	45·8	46·9	45·3
Cl (m-equiv/kg)	36·6	41·8	38·7	37·5	40·6
N (g/100 g)	1·51	1·47	1·46	1·44	1·61
N (mg/m-equiv K)	20·0	15·9	21·0	19·5	21·3
Liver					
Weight (% of body wt.)	2·20	2·24	4·24	4·52	2·29
Water (g/100 g)	80·1	80·0	76·4	77·3	80·9
K (m-equiv/kg)	80·9	82·5	70·2	75·6	70·7
Na (m-equiv/kg)	47·1	41·0	41·8	39·9	54·3
Cl (m-equiv/kg)	50·5	46·5	42·4	39·5	53·7
N (g/100 g)	2·28	2·23	1·63	1·55	2·28
N (mg/m-equiv K)	28·2	27·0	23·2	20·5	32·3
Heart					
Weight (% of body wt.)	0·72	0·68	0·73	0·68	0·64
Water (g/100 g)	83·2	82·9	82·9	82·9	83·1
K (m-equiv/kg)	77·2	84·3	80·3	78·4	82·1
Na (m-equiv/kg)	45·2	40·2	42·3	43·6	45·1
Cl (m-equiv/kg)	40·0	46·7	38·8	42·3	39·7
N (g/100 g)	1·89	1·94	1·80	1·80	1·81
N (mg/m-equiv K)	24·5	23·0	22·3	23·0	22·1

DISCUSSION

Although there are minor points of uncertainty the main results are clear, and only these will be discussed.

Electrolyte balances

Chloride ions were freely excreted by the new-born piglet unless the accompanying cation was retained, and the kidney of the new-born piglet has a much greater capacity for excreting potassium ions than sodium ions. The work of Tudvad, McNamara & Barnett (1954) and of McCance & Widdowson (1957) suggests this is true also of the human infant. Nevertheless, the potassium ions were not completely excreted, and rose to concentrations in the extracellular fluid which were toxic in the group of animals which received the solutions of potassium chloride in water. According to Black (1957) 'an absolute increase in the K content of the body is probably rather uncommon, and has not been adequately demonstrated'. These experiments would appear to have done so if it has not been done before, for the animals which were *not* fed retained 7 m-equiv more K/kg/24 hr than their controls, and those which *were* fed 4 m-equiv. The administration of potassium chloride brought about no increase in the total amount of water in the body or changes in the over-all metabolism of protein. There can hardly have been any expansion of the extracellular fluids and these could have accommodated less than 1 m-equiv K/kg body wt./24 hr. Since, moreover, the retentions of potassium considerably exceeded those of chloride we may infer that most of the potassium was taken up by the cells. In the skeletal muscles this was certainly so. If all the chloride in this tissue is assumed to be extracellular it may be calculated from the figures given in Table 3 that 14·3 of the 16·3 m-equiv of surplus potassium found in each kg of the skeletal muscles of the animals given the potassium chloride in water were in the cells and 4·4 of the 4·8 m-equiv of the surplus potassium found in each kg of the skeletal muscles of the animals given the potassium chloride in the milk were likewise in the cells. The skeletal muscles, moreover, accounted for a large fraction of the surplus potassium retained by the whole animal, but exact calculations cannot be made. Storage in this site is generally regarded as the fate of most of the potassium retained by the body after its administration (Fenn, 1940; Fenn, Noolan, Mullins & Haege, 1941–2; Grob, Johns & Liljestrand, 1957; Grob, Liljestrand & Johns, 1957). The capacity to absorb and retain potassium in excess of its anticipated requirements has been encountered by M. O. Beem & C. A. Smith (personal communication) when the intakes have been raised and the present experiments show in an exaggerated form the internal changes which probably took place.

The blood sugars

The animals given the potassium chloride in water had high concentrations of sugar in the blood when they were killed after 40 hr starvation. This is a finding worth remark, for unfed new-born piglets tend to become hypoglycaemic, especially if they are cold (Goodwin, 1957). Hyperglycaemia does not seem to have been recorded in patients suffering from paralysis due to potassium intoxication, perhaps because estimations of the blood sugar have not been made before giving the large amounts of glucose which now constitute part of the treatment (Winkler, Hoff & Smith, 1941; Finch, Sawyer & Flynn, 1946; Govan & Weiseth, 1946; Merrill, Levine, Somerville & Smith, 1950; Bull, Carter & Lowe, 1953; Nadler, 1953; Gamstorp, Hauge, Helweg-Larsen, Mjönes & Sagild, 1957). This observation of raised blood sugar makes one think of the hyperglycaemia reported by Silvette (1938) and his associates (Silvette, Britton & Kline, 1938) to follow injections of potassium salts. Since these authors emphasized the difference they had found between the responses of different species, this may explain why the effect has not been noted in human beings, and why Odashima (1931–2) did not observe it in rabbits. The only experiments on man with any bearing on this matter seem to be those of Thompson & McQuarrie (1934), who found in controlled work on a diabetic child that heavy doses of potassium chloride greatly increased the output of glucose in the urine. Before this time doses of potassium salts had been claimed to lower the blood sugar in diabetes (Semler, 1925), but the results obtained were quite unconvincing. The rise in blood sugar may be due to the effect of potassium salts on the liberation of adrenalin (Fenn, 1940). It seems less likely to be due to the kind of effects which have been observed in tissue slices or portions of rat's diaphragm respiring *in vitro* in media containing concentrations of potassium far above the physiological ranges possible in an extracellular fluid during life (Clarke, 1955; Ashmore, Cahill, Hastings & Zottu, 1957; Cahill, Ashmore, Zottu & Hastings, 1957).

Homoeostasis and growth

The importance of growth as an agent in homoeostasis during the new-born period has been demonstrated and discussed before (McCance & Widdowson, 1956, 1957), but mainly as it applies to nitrogen metabolism. The present results show its importance in another way. The animals getting the potassium in their milk were able to grow, and although this did not prevent some rise taking place in the potassium in their sera and muscle cells, they grew perfectly normally so far as could be observed, and exhibited no signs of paralysis or the high blood sugars so obvious in the animals given the same amount of potassium but without the means by which to grow. It is worth noting in this connexion that when the potassium was given in water the retention of 2·9 m-equiv K/kg starting weight produced paralysis and widespread signs of toxicity in 40 hr, whereas the same amount of potassium in milk was followed by a retention of 10·5 m-equiv K/kg and signs of good health. It is possible that withholding all food on the eleventh day while maintaining the infusion containing the potassium salts was what precipitated the potassium intoxication in the infant reported by Govan & Weiseth (1946). There may be other examples of this kind of thing in the literature but it is unnecessary to labour the point. It is evident that potassium infusions should be given with greater care in a starving than in a growing infant, and the protection afforded by food may not be confined to its effects on growth, for the storage of glycogen at all ages is accompanied by the deposition of potassium and water (Calkins, Taylor & Hastings, 1954) and greater care should therefore be taken over potassium infusions in adults if they are being given without, rather than with, glucose.

SUMMARY

1. Piglets have been given the following by stomach tube two hourly for the first forty hours of their lives (1) water, (2) water and KCl, (3) sow's milk, (4) sow's milk and KCl, and (5) nothing.

2. Most of the KCl was excreted, but the administration of KCl in water led to progressive retention of K, paralysis and raised blood sugar.

3. The animals getting KCl in milk retained a mild excess of K but growth, which was normal, completely protected them from toxic effects.

4. The serum, urines and tissues have been collected and analysed and the results reported in tabular form.

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THE DISTRIBUTION OF CHOLINESTERASE IN THE ADRENAL GLANDS OF THE RAT, CAT AND RABBIT

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Cholinesterase has been detected in many different tissues and is especially abundant in nervous tissue and in structures which receive a rich autonomic innervation. Ord & Thompson (1950) observed that true cholinesterase predominates in organs in which acetylcholine exerts a nicotine-like action, while where the muscarine-like effect is dominant both true and pseudocholinesterase occur. Using manometric methods for the determination of cholinesterase activity, Antopol & Glick (1940) reported cholinesterase activity in the adrenal medulla of the rabbit: later Ord & Thompson (1950) found that the medullary enzyme was predominantly of the true variety, and Hagen (1955) reported that activity was mainly confined to the microsomal fraction of the homogenized gland. Sawyer & Everett (1947) described true cholinesterase in the rat's adrenal cortex.

A method for the histochemical localization of cholinesterase activity was introduced by Koelle & Friedenwald (1949), and has since been modified (Koelle 1951; Couteaux, 1951; Coërs, 1953; Snell & McIntyre, 1956; Coupland & Holmes, 1957). Koelle (1950) reported the presence of both true and pseudocholinesterase in the cat's adrenal, the true being confined to nerve fibres, the pseudo being found in the cells of the zona glomerulosa and in the chromaffin cells. Later, however, Koelle (1951) attributed the previously reported positive reaction of the chromaffin cells to diffusion effects, and concluded that the chromaffin cells had a negative reaction. It is apparent from the investigations of Hawkins & Mendel (1947) and Koelle (1955) that there is a species difference in the relative amounts of true and pseudocholinesterase found in any tissue. It has, however, been found that, by applying methods which will reveal both the true and pseudo-enzyme (Koelle, 1955; Coupland & Holmes, 1957), nerve fibres in many different situations may be clearly demonstrated.

In this work a histochemical method for demonstrating the localization of cholinesterase has been applied to the adrenal glands of the rat, cat and rabbit. The method has been found to give a clear and reproducible picture of the distribution of nerve fibres in both cortex and medulla, and the findings have been compared with those obtained by other methods.

Although it is now generally accepted that the adrenal medulla is innervated by preganglionic sympathetic nerve fibres (Hollinshead 1936; Hollinshead & Finkelstein, 1937; Young, 1939) opinions differ with regard both to the innervation of the adrenal cortex and to the method of termination of nerve fibres in the gland. Using silver impregnation techniques, Dogiel (1894), Willard (1936), Kiss (1951) and Sarter (1954) observed nerve plexuses and apparent endings in both the cortex and medulla of a variety of different animals, including the cat, dog, rabbit and monkey. Lever (1953), using a methylene-blue technique, observed fine beaded nerve fibres which were in intimate relation with the cells of the zona glomerulosa of the rat. Swinyard (1937) and MacFarland & Davenport (1941) were, however, unable to find any evidence in favour of cortical innervation in the rat and other mammals.

The mode of termination of the nerve fibres in the adrenal medulla has been described as being by terminal end-bulbs (Hollinshead, 1936), terminal arborization about chromaffin cells (Swinyard, 1937; MacFarland & Davenport, 1941) and as a nervous 'ground plexus' without discrete endings (Hillarp, 1946). Willard (1936), although reporting the presence of *boutons termineaux* and *boutons de passage* in relation to chromaffin cells, considered that these were insufficient in number to justify the conclusion that they are the main agents for stimulating the secretory cells.

MATERIAL AND METHODS

Adult cats, rats (Sprague-Dawley strain) and rabbits have been used. The technique described previously (Coupland & Holmes, 1957) was used for revealing sites of cholinesterase activity. The adrenal glands were removed immediately after death and fixed for 4–16 hr in 10% formol-saline. Frozen sections were then cut at thicknesses of 10–50 μ, washed in isotonic saline for 30 min and finally mounted on slides and allowed to dry. They were then incubated at 37° C for 4–24 hr in:

0·6 ml. copper-glycine (glycine 3·75 g; $CuSO_4.5H_2O$ 2·5 g, distilled water to 100 ml.);
0·6 ml. magnesium chloride soln. ($MgCl_2.6H_2O$ 9·52 g, distilled water to 100 ml.);
5 ml. 0·2 M acetate buffer, pH 5 or 5·6;
7·6 ml. 40% sodium sulphate in water;
1·2 ml. acetylthiocholine (for localization of true cholinesterase) or butyrylthiocholine (for pseudocholinesterase) made up according to the method of Koelle (1951).

After incubation the sections were washed for 2–5 min in running water and then treated with either dilute ammonium sulphide or with ammonium sulphide saturated with copper sulphide for 30 sec. A final 10 min wash in water was followed by dehydration in alcohols and mounting in Canada Balsam.

Some sections were counterstained with safranine, thionine, or haematoxylin. Unstained sections were examined by phase contrast. The optimum pH for incubation varies with species: with the rabbit best results were obtained at pH 5·6, while with the cat and rat pH 5 was most satisfactory. The use of a higher pH results in a more general positive reaction due to non-specific tissue esterases, which tend to obscure the nervous elements. The presence of sodium sulphate in the incubating solution results in the formation of a finer precipitate than occurs with the Coërs (1953) method and hence allows more accurate localization.

Control preparations were treated with eserine in a final concentration of 10^{-5} M, or with di-isopropylfluorophosphate (DFP) 10^{-6} or 10^{-7} M, for cat and rat, and $0{\cdot}5 \times 10^{-6}$ M for the rabbit. Sections to be treated by DFP were immersed (after attaching to slides) in a solution of DFP in isotonic saline for 20 min, after which they were rinsed in saline for 20 min and finally placed in the incubating solution. The other inhibitor was added to the incubating solutions.

Sections of rabbit adrenal impregnated with silver, by both the Nonidez (1939) method and by the Holmes (1947) technique, have also been examined and compared with the above material.

RESULTS

Rat

The connective tissue element of the capsule shows a strong positive reaction using butyrylthiocholine (Pl. 1, figs. 2, 3), which is abolished by pre-treatment with 10^{-7} M DFP and 10^{-5} M eserine and is considered to be due to pseudocholinesterase. Cortical cells and blood vessels are all negative. Chromaffin cells give a patchy slight positive reaction for true cholinesterase when tissues are incubated for more than 5 hr, but with shorter times only nerve fibres gave a positive reaction. Nerve fibres give a strong positive reaction after incubating sections in either acetyl- or butrylthiocholine for 3 hr; the reaction is completely inhibited by 10^{-5} M eserine, but only slightly inhibited by 10^{-6} M DFP and is, therefore, considered to be due mainly to the presence of true cholinesterase, although a small amount of the pseudocholinesterase is also present.

Nerve fibres are observed passing through the periadrenal connective tissue to reach the capsule. These fibres give a positive reaction for both true and pseudocholinesterase, the true variety predominating. On reaching the capsule of the gland branches may be traced to a capsular plexus (Pl. 1, fig. 1), while the majority of fibres penetrate the capsule and pass on in large discrete bundles to the medulla. From the capsular plexus cholinesterase-positive fibres may be traced into the zona glomerulosa (Pl. 1, figs. 2, 3), where they branch and run in intimate association with both cortical cells and blood vessels. Small arteries observed entering the gland are often encircled by fine nerve fibres.

On reaching the medulla the bundles of nerve fibres divide and branches may be traced into a three-dimensional cholinesterase-positive meshwork (Pl. 1, fig. 4) which pervades the whole region. Collections of ganglion cells (Pl. 1, figs. 4, 5) are often observed in the medulla. The cytoplasm of the neurones gives a positive reaction for true cholinesterase. The neurones are surrounded by a felt work of cholinesterase-positive nerve fibres and satellite cells. The latter give a positive reaction for pseudocholinesterase. The true continuity between typical nerve bundles and the medullary mesh can readily be demonstrated in serial sections and makes the nervous nature of this meshwork beyond doubt. Its appearance is, however, somewhat different from the more conventional picture revealed by silver impregnation methods by virtue of the fact that both nerve axis cylinders and associated cellular elements give a positive reaction for cholinesterase. This subject will be discussed more fully below.

The nervous elements in the zona glomerulosa and in the medullary nerve mesh are both coarse and fine. The coarser fibres in both situations are occasionally associated with large ovoid nuclei which are typical of Schwann cell nuclei (Pl. 1, fig. 6) and are so named in this work. These nuclei are readily observed by suitable counterstains or by phase contrast, but may appear as large varicosities in the unstained preparation when examined by the ordinary-light microscope. The finer fibres show small ovoid varicosities at intervals (Pl. 2, fig. 7). These are characterized by having a cholinesterase-negative centre. Exceptionally, a fine strand of the medullary mesh or a cortical fibre appears to terminate in such a swelling (Pl. 2, fig. 8). Although the general arrangement of the nervous structures may be revealed by incubation in substrates containing either acetyl- or butyrylthiocholine, the finer strands give a positive reaction for true cholinesterase but not the pseudo-enzyme and are only observed after incubation in acetylthiocholine. The smooth muscle present in the wall of the adrenal vein gives a positive reaction for pseudocholinesterase.

Cat

Capsular cells give a negative reaction for cholinesterase. Cortical cells of the zona glomerulosa give a strong positive reaction for pseudocholinesterase (Pl. 2, figs. 9, 10). Other cortical cells give a negative reaction. In sections incubated for less than 4 hr the chromaffin cells give a negative reaction; the slight patchy positive reaction which appears after longer incubation is considered to be the result of diffusion.

Nerve fibres give a positive reaction for both true and pseudocholinesterase after 3 hr incubation. They may be traced from the surrounding tissues to the surface of the gland. On reaching the adrenal some fibres enter the gland and run as discrete bundles through the cortex to the medulla (Pl. 2, fig. 9), where they divide and become continuous with a cholinesterase-positive nervous mesh or plexus (Pl. 2, fig. 11). Other nerves form a subcapsular plexus from which fibres may be traced into the zona glomerulosa (Pl. 2, fig. 10); these run in close association with cortical cells and blood vessels. As in the rat, some fibres encircle capsular arteries. The finer cholinesterase-positive nerve fibres in both cortex and medulla show small and large varicosities. The centres of the small varicosities give negative reactions for cholinesterase, the large varicosities are produced by cell nuclei. The finer strands of the medullary mesh are revealed by acetylthiocholine, but not by butyrylthiocholine; hence, as in the rat, pseudocholinesterase is mainly confined to the main nerve bundles and coarser strands of the medullary plexus.

Groups of sympathetic neurones are occasionally observed in contact with the capsule of the gland. The cytoplasm of these cells gives a negative reaction for cholinesterase. A positive reaction for both true and pseudocholinesterase is observed over the cell membrane. Satellite cells give a positive reaction for pseudocholinesterase.

Smooth muscle cells in the wall of the adrenal vein give a positive reaction for pseudocholinesterase.

Rabbit

Both capsular and cortical cells give a negative reaction for cholinesterase. Chromaffin cells give a negative reaction in sections incubated for less than 6 hr. Sections incubated for longer periods give a patchy slight positive reaction for true cholinesterase which is considered to be a diffusion effect. Very occasionally isolated neurones are observed in the medulla; the cytoplasm of these cells gives a positive reaction for the true enzyme. Smooth muscle cells lying in the wall of the adrenal vein give a positive reaction for pseudocholinesterase.

Nerve fibres give a positive reaction after 3 hr incubation, which is mainly due to the presence of true cholinesterase. Nerves may be traced to the surface of the gland, and these then penetrate the capsule. Branches may be traced to a subcapsular plexus, but the majority of fibres pass on to the medulla where they join a cholinesterase-positive nervous mesh (Pl. 2, fig. 12). From the subcapsular region a few fibres pass centrally among cortical cells and blood vessels of the glomerular zone and unite with adjacent nerves to form a plexus (Pl. 3, fig. 13). Small bundles then run on to the medulla. A few fine nerve fibres may be observed running between the cells of the zona glomerulosa, but they are less numerous than in the other animals examined. As in the rat and cat, the cholinesterase-positive medullary strands show large and small varicosities. The large varicosities are produced by ovoid nuclei and the small ones have a central negative cholinesterase reaction (Pl. 3, figs. 14, 15).

The arrangement of nerve fibres in the adrenal gland of the rabbit, as revealed by silver impregnation techniques, has been compared with the pattern observed after the application of the cholinesterase reaction. The general pattern of fibres is similar in the two instances (Pl. 3, figs. 16, 17); nerve bundles of similar size may be observed breaking up into numerous smaller branches which pass between the chromaffin cells. The silver-impregnated fibres may be followed for a variable distance through the medulla; varicosities are rare but are occasionally observed. Some fibres apparently form complete loops (Pl. 3, fig. 18), while others may end as a slight swelling in the neighbourhood of a chromaffin cell. A plexus of anastomosing strands of cholinesterase-positive material exists in the medulla and is directly continuous with the nerve bundles which have penetrated the cortex. Free endings are rarely observed (Pl. 3, fig. 15). Many of the strands of the network have a greater diameter than comparable silver-impregnated fibres and this fact, together with the presence of associated nuclei which morphologically resemble those of Schwann cells, suggests that the picture obtained by the cholinesterase technique is a composite one produced by both axis cylinders and associated cellular elements. The finer strands of the cholinesterase-positive mesh have a similar diameter to the fibres revealed by silver impregnation; they give a positive reaction for the true enzyme only. Small varicosities are observed along the strands; these are comparable in size with the *boutons* described by earlier workers (Willard 1936) and observed in some of our own silver preparations. The similarity in thickness between fine cholinesterase-positive nerve strands and silver-impregnated axis cylinders indicates that the associated cellular elements must be reduced to a minimum in relation to these strands. This view is supported by the finding that the finer elements of the nervous mesh give only a positive reaction for true cholinesterase, while the coarser strands which are associated with Schwann cell nuclei give a reaction for both true and pseudo-enzymes. It would therefore seem that the pseudocholinesterase reaction is mainly confined to the Schwann cell cytoplasm.

DISCUSSION

Although true cholinesterase has been reported in a number of non-nervous tissues (Sawyer & Everett, 1947), in the present work this enzyme has been demonstrated only in the nervous elements of the adrenal gland. A positive reaction for pseudocholinesterase has been observed in the capsule of the rat adrenal, the cortical glomerulosa zone cells of the cat and in the smooth-muscle cells of the adrenal vein in all three animals. Endothelial cells and reticular fibres give a negative reaction in all instances.

In all three animals chromaffin cells give a negative reaction for both true and pseudocholinesterase providing the sections are not incubated for more than 5 hr; a period sufficient to produce an intense reaction in nervous elements. After more prolonged incubation, a slight positive reaction for true cholinesterase is observed and is considered to be a diffusion artifact. As a result of these findings, it is possible that the true cholinesterase activity reported by Hagen (1955) in the microsomal fraction of the homogenized ox adrenal may have been due to the inadvertent inclusion of nerve fibres, and it appears that in the rat, cat and rabbit the enzyme concerned in acetylcholine metabolism is not only not in a different part of the chromaffin cell from the large-granule elements, but is actually located outside the cell membrane. This finding is in keeping with the observation of Blaschko, Hagen & Welch

(1955) that acetylcholine will not liberate pressor amines from the large-granule fraction of the ox-adrenal. The localization of the enzyme external to the chromaffin cell would, however, imply that the signal, whether chemical or physical, which results from the release of acetylcholine in the neighbourhood of the chromaffin cell, must cross first the cell membrane before reaching the ergastoplasm and finally the pressor amine-containing mitochondria.

The association between nerve fibres and cells of the zona glomerulosa in the present work is in keeping with the findings of Dogiel (1894), Willard (1936), Evans (1947), Kiss (1951), Lever (1953) and Sarter (1954). In all three animals a capsular or subcapsular nerve plexus is observed. From this a few fibres pass into the zona glomerulosa where they branch and run in intimate contact with cortical cells and capillaries. These glomerular zone fibres are both coarse and fine; some are associated with ovoid nuclei (Pl. 1, fig. 6) which are considered to be Schwann cell nuclei. The finer fibres show small varicosities (Pl. 2, fig. 7) which are identical with those observed on the fine strands of the medullary nerve mesh (Pl. 3, figs. 14, 15) and which may be equivalent to the *boutons de passage* observed by Willard (1936). Owing to the intimate contact of the fine nerve fibres with both the cortical cells and blood vessels, it has not been possible to decide whether they are primarily concerned with the innervation of the cells of the zona glomerulosa or with vascular responses. The small arteries and arterioles observed in the capsule and entering the outer aspect of the cortex are, however, often richly innervated.

Nerve plexuses have not been seen in the fascicular and reticular zones of the cortex. Nerve fibres in these regions are apparently in transit to the medulla and the fine varicose fibres, which are a feature of the zona glomerulosa and medulla, have not been observed.

The majority of nerve fibres penetrate the capsule and run in large discrete bundles to the medulla where they branch and join a cholinesterase-positive meshwork or plexus. Using a silver impregnation technique, Dogiel (1894) reported that nerve fibres were mainly related to the arterial poles of the chromaffin cells; in the present work a similar relationship between the chromaffin cells and the nervous strands has been observed and, although filaments may be seen passing between the chromaffin cells towards a venous sinus, they have not been found in the subendothelial tissue of the sinuses.

The cholinesterase-positive medullary mesh is composed of strands of variable thickness and is in continuity with nerve fibres which may either have run directly to the medulla or have taken part in the formation of the subcapsular or glomerular zone plexus before passing on to the centre of the gland. The coarser strands of the net are occasionally associated with Schwann cell nuclei, and it is likely that the structure revealed by the enzyme reaction is a composite element of both axis cylinders and supporting Schwann cell cytoplasm. The findings are, therefore, in keeping with the work of Hillarp (1942), who described a terminal nervous ground plexus 'of axons running in a fine-meshed network of anastomosing strands formed by Schwann cell plasmodium'. The finer strands of the medullary mesh are seen only after incubation in acetylthiocholine and are comparable in thickness with the finest fibres revealed by silver impregnation techniques. Schwann cells have not been observed in association with these structures after incubation with either acetyl- or butyrylthiocholine and, therefore, it appears that in this position the supporting tissue must be absent, reduced to a minimum or have no pseudocholinesterase activity. Small ovoid varicosities, in which the central region gives a negative cholinesterase reaction, are frequently observed along the course of these fibres; they are comparable in size and shape with *boutons de passage* occasionally observed in silver preparations, but are more numerous. Although a few strands of the medullary nerve mesh apparently end discretely with a terminal dilatation which may be likened to a *bouton* or end bulb, these apparent endings are rarely observed and are considered to be due to the inadvertent cutting of individual strands of the meshwork adjacent to ovoid dilatations.

Snell (1957) showed that, by reducing the pH of the incubating solution to 4·2, axis cylinders may be selectively demonstrated in the superior cervical ganglion of the cat. This method has not been successful in the present work, and as the final picture is a result of the cholinesterase activity of both axis cylinder and supporting tissues the findings do not give any information on the question of the mode of termination of the axis cylinder.

SUMMARY

1. The adrenal capsule of the rat and the cortical cells of the zona glomerulosa of the cat give a positive reaction for pseudocholinesterase.

2. Chromaffin cells give a negative reaction for both true and pseudocholinesterase.

3. Nerve fibres give a positive reaction for both types of enzyme, the true variety preponderating.

4. Nerve fibres form plexuses in the capsular or subcapsular regions and in the zona glomerulosa.

5. Large bundles of nerve fibres may be traced into a medullary nervous meshwork. A few ganglion cells may be observed in the medulla of all three species: they are most numerous in the rat.

6. Small ovoid varicosities exist along the nerve strands. The centres of these structures give a negative cholinesterase reaction.

7. In the adrenal gland the cholinesterase technique demonstrates both axis cylinders and supporting elements.

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EXPLANATION OF PLATES

Scale marks indicate 0·1 mm unless otherwise labelled.

Plate 1

Fig. 1. Nerve fibres in capsule of the rat adrenal gland. Tissue incubated 4 hr; substrate acetylthiocholine.

Fig. 2. Strong positive reaction in capsule of rat adrenal. Nerve fibres pass between cells of the zona glomerulosa and a few then enter the zona fasciculata and run towards the medulla. Substrate acetylthiocholine; incubated 18 hr.

Fig. 3. Rat adrenal showing capsule (positive reaction), zona glomerulosa and outer part of zona fasciculata (negative reaction). Nerve fibres pass from the subcapsular region into the zona glomerulosa. Substrate acetylthiocholine; incubated 18 hr.

Fig. 4. Rat adrenal medulla showing nerve net and small collection of ganglion cells (*G*). Chromaffin cells give a faint positive reaction. Substrate acetylthiocholine; incubated 20 hr.

Fig. 5. Collection of neurones in rat adrenal medulla. A positive reaction occurs throughout the cytoplasm of the nerve cells. Pre-treated with DFP; substrate acetylthiocholine; incubated 20 hr.

Fig. 6. Small nerve bundle in the glomerular zone of the rat adrenal cortex with an associated Schwann cell nucleus. Substrate acetylthiocholine; incubated 4 hr.

Plate 2

Fig. 7. Fine nerve fibres in glomerular zone of rat adrenal. Arrows indicate small ovoid varicosities on cholinesterase positive strands. A Schwann cell nucleus (*S*) lies at the point of junction of two fibres. Substrate acetylthiocholine; incubated 4 hr.

Fig. 8. Fine strands of the cholinesterase-positive medullary mesh of the rat showing small varicosities and an apparent terminal swelling. Section, 10 μ thick, incubated in acetylthiocholine for 4 hr.

Fig. 9. Cat adrenal. Positive cholinesterase reaction in the zona glomerulosa, medulla, and in nerve fibres; a few fibres may be seen in the capsular region and a large bundle penetrates the gland. Substrate acetylthiocholine; incubated 20 hr.

Fig. 10. Cat adrenal showing an indentation of cortex with a positive cholinesterase reaction in the cells of the zona glomerulosa and in nerve fibres. Nerves can be traced from the capsular region (*C*) into the zona glomerulosa. The cells of the zona fasciculata (*F*) are negative. Substrate acetylthiocholine; incubated 20 hr.

Fig. 11. Nerve mesh in cat adrenal medulla. The clear area on the right of the figure is the zona reticularis. Substrate acetylthiocholine; incubated 20 hr.

Fig. 12. Cholinesterase-positive medullary mesh in rabbit. *V* = venous sinus. Substrate acetylthiocholine; incubated 5 hr.

Plate 3

Fig. 13. Cholinesterase-positive nerve fibres in the subcapsular region and glomerular zone of the rabbit adrenal. Substrate acetylthiocholine; incubated 8 hr.

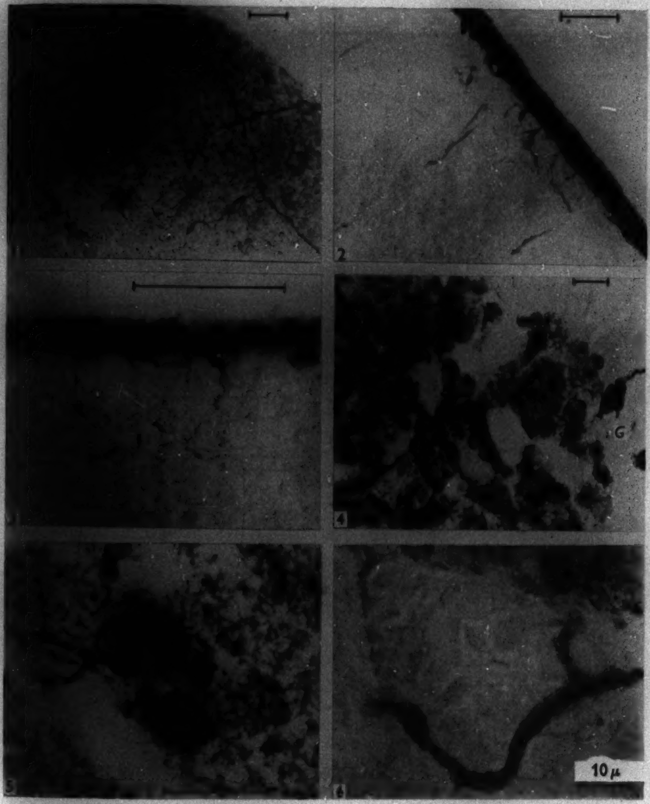
Fig. 14. Fine cholinesterase-positive strands in the medulla of the rabbit. The centres of small ovoid varicosities give a negative reaction. Substrate acetylthiocholine; incubated 4 hr.

Fig. 15. Rabbit adrenal medulla. Apparent ending of fine cholinesterase-positive strand showing a terminal swelling which has a cholinesterase-negative centre. Substrate acetylthiocholine; incubated 4 hr.

Fig. 16. Nerve fibres in a rabbit's adrenal medulla as revealed by the cholinesterase technique. Substrate acetylthiocholine; incubated 5 hr.

Fig. 17. Nerve fibres in a rabbit's adrenal medulla revealed by silver impregnation.

Fig. 18. Fine silver-impregnated nerve fibre apparently forming a loop in the adrenal medulla of a rabbit.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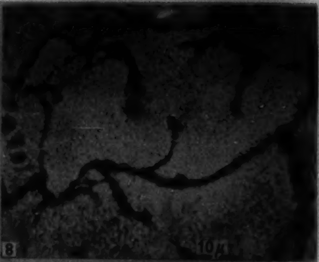
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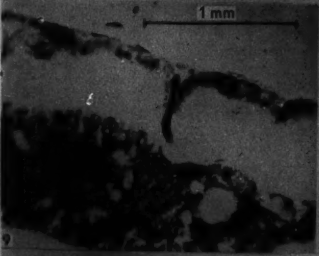
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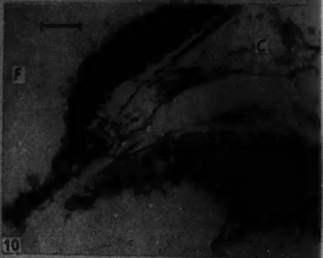
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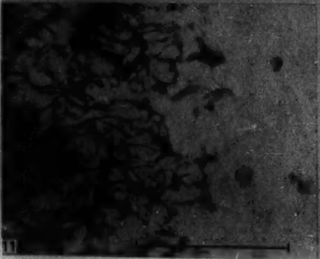
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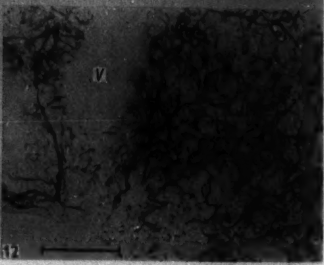
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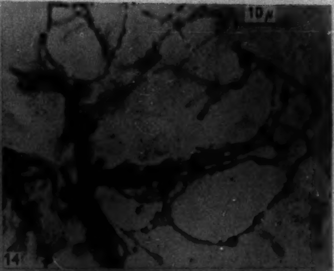
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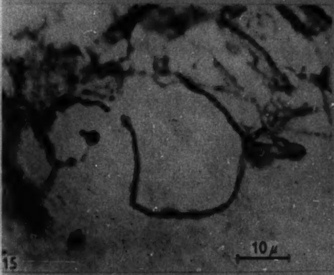
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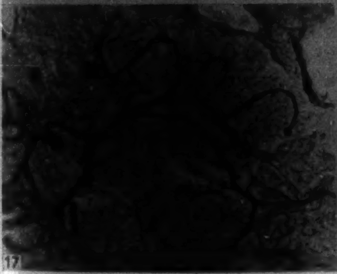
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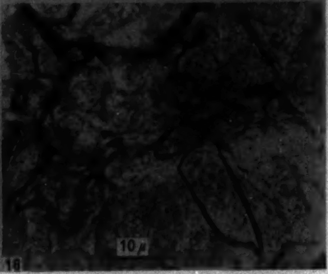
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THE DESTRUCTION OF THE ANTIDIURETIC ACTIVITY OF VASOPRESSIN BY —SH ACTIVE COMPOUNDS

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It has been shown previously that the incubation of vasopressin with kidney homogenates results in the inactivation of its antidiuretic activity, and it was suggested that the inactivation was enzymic in character. Fractionation of kidney homogenates indicated a greater concentration of the enzymic activity in the 'particle-free' supernatant fluid of the homogenate than in the particulate elements of the cell (Dicker & Greenbaum, 1956*a*). The present investigation was undertaken with a view to securing more information on the mechanism of inactivation of vasopressin by the 'particle-free' supernatant fluid. A preliminary account of this work has been published previously (Dicker & Greenbaum, 1956*b*).

METHODS

Pig's kidneys, fresh from the slaughterhouse, were homogenized in 0·25 M sucrose solution as described previously (Dicker & Greenbaum, 1956*a*). The 'particle-free' supernatant fluid was obtained after centrifugation at 20,000 *g* for 30 min.

The assays of antidiuretic activity were carried out on rats under ethanol anaesthesia, following Dicker's (1953) method as described by Dicker & Greenbaum (1954).

The sulphydryl content of kidney homogenates was estimated as follows: 0·5 ml. of the fraction was incubated with 3 ml. of 0·005 M *p*-chlormercuribenzoate solution in the presence of 4 ml. of 0·2 M acetate buffer pH 5·3 at 37° C for 30 min, after which the mixture was dialysed against 1% NaCl solution for 3 days. The entire content of the sac was then digested in a mixture of 5 ml. concentrated H_2SO_4 and 5 ml. concentrated HNO_3 for 24 hr. The protein-bound Hg was estimated by the dithizone method described by Ballentine & Burford (1957) for the estimation of Cu^{2+}.

Drugs. Vasopressin (Pitressin; Parke, Davis) was used for the incubation with tissue homogenates and as standard for the estimation of antidiuretic effects. The antidiuretic activity of the drug Pitressin has been referred to throughout as 'vasopressin'.

RESULTS

Fractionation of the 'particle-free' supernatant fluid

In an attempt to isolate the enzymic activity, $(NH_4)_2SO_4$ was added to the ice-cold supernatant to a concentration of 30% saturation. After this had stood for 30 min the precipitate was collected by centrifugation and more $(NH_4)_2SO_4$ was then added to the supernatant fluid to bring it to 40% saturation. This procedure was then repeated to obtain 50 and 60% saturations. The precipitate from each of these stages was dissolved in water and dialysed for 36 hr at 4° C until free of sulphate. The volume was then adjusted so that 1 ml. of the fraction was equivalent to 0·1 g fresh tissue. The fractions so obtained were incubated with 10 m-u. vasopressin for 30 min at 37° C, acidified with acetic acid and boiled for 3 min (Dicker & Greenbaum, 1956*a*); the supernatant was then assayed for its antidiuretic activity. It will be seen from Table 1 that all fractions inactivated vasopressin to some extent; the greatest inactivation occurred with the fraction which had been precipitated between 40 and 50% saturation with $(NH_4)_2SO_4$.

TABLE 1. Amounts of antidiuretic activity destroyed by fractions of supernatant fluid from kidney homogenates

Degree of saturation with $(NH_4)_2SO_4$ (%)	Amount of activity destroyed (m-u.)
0	3·0
30–40	5·1
40–50	6·4
50–60	4·9

Initial amount of vasopressin at time 0, 10 m-u. in 5 ml. Krebs–Ringer solution. Results are means of 3 experiments. Volume of each fraction tested: 0·5 ml.; time of incubation, 30 min.

The prolonged dialysis to which the fractions were submitted may have led to a loss of enzymic activity. To ascertain whether it would be possible to dispense with dialysis, it was necessary to show that the presence of a small amount of residual $(NH_4)_2SO_4$ did not interfere with the accuracy of assays. As a matter of fact, the addition to vasopressin of amounts of $(NH_4)_2SO_4$ six times greater than those normally present in the centrifuged pellet (i.e. before dialysis of the precipitated fractions) had no effect on the accuracy and validity of the antidiuretic assay. In a series of four assays, each of three groups, the percentage recovery varied between 87 and 110, with fiducial limits of error between 79 and 127%, which agreed very well with the normal limits set for the assay (Dicker & Greenbaum, 1956*a*). This finding made it possible to carry out all further experiments without dialysis.

In further attempts at a crude fractionation, the 'particle-free' supernatant fluid was adjusted to pH values 4, 5, 6, 7 and 8 before the addition of $(NH_4)_2SO_4$. The fraction precipitating between 40 and 50% saturation was collected in each case. The precipitates so obtained were dissolved in water so that 1 ml. of the fraction was equivalent to 0·1 g fresh tissue. Except for the fact that they were not dialysed, these fractions, called herein F4, F5, F6, F7 and F8, were treated in the same way as the previous ones and assayed. In all six experiments, fractions precipitated at pH 4, 5, 7 and 8 inactivated vasopressin to some degree, whereas that precipitated at pH 6 did not (Table 2). The fraction obtained at pH 8 was the most active (Fig. 1). It was also observed that in every case the total amount of vasopressin inactivated by the fractions separately exceeded the amount of drug inactivated by the original supernatant fluid from which they were obtained (Table 2). When all fractions, however, were combined before incubation, the amount of vasopressin inactivated by them was similar to that inactivated by the original supernatant fluid. These findings suggested either that the original supernatant fluid contained an inhibitory factor or that one of the fractions obtained after precipitation with ammonium sulphate was inhibitory in the presence of the others, or both. It will be seen from Table 2 that while fractions F5 and F7 separately inactivated vasopressin to some degree, the addition of F6 to either of these fractions inhibited their capacity to inactivate the drug.

TABLE 2. Inactivation of vasopressin by fractions of kidney homogenate precipitated at different pH values. All fractions were precipitated at between 40 and 50% $(NH_4)_2SO_4$ saturation

Fraction	Amounts of antidiuretic activity destroyed (m-u.)	
F4	2·0	2·4
F5	2·2	2·8
F6	0·0	0·0
F7	2·5	2·6
F8	3·7	3·1
Sum	10·4	10·9
F5+F6	0	0
F6+F7	0	0
F5+F7	2·3	2·7
S	6·0	5·8

Fractions 4–8 corresponded to pH values 4–8. S: original cell-free supernatant fluid. Initial amount of vasopressin, 10 m-u. in 5 ml. Krebs–Ringer solution; volumes of fractions 4 to 8 and of S, 0·5 ml., corresponding to 0·05 g fresh tissue; time of incubation, 30 min. Results are means of three experiments each.

In view of the complexity of the material under investigation it was decided to limit the present work on the mechanism of inactivation of vasopressin to the study of one of the fractions only. The fraction chosen was that which is precipitated between 40 and 50% saturation with $(NH_4)_2SO_4$ at pH 8, the fraction F8.

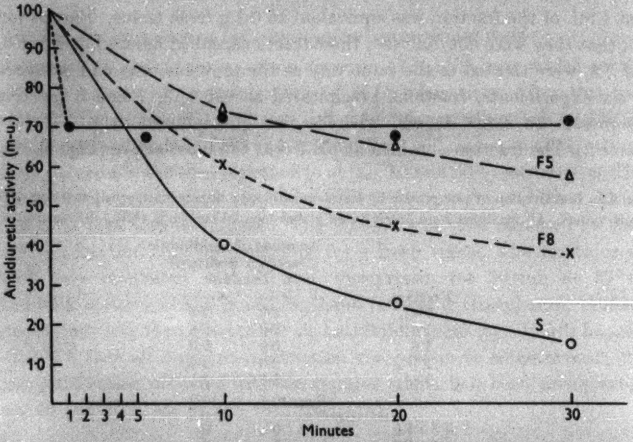

Fig. 1. Comparison between rates of inactivation of vasopressin by fractions of kidney homogenate. Initial amount of vasopressin: 10 m-u. in 5 ml. Krebs–Ringer solution. S; original cell-free supernatant fluid. F5; fraction obtained by precipitating S at pH 5 with 40–50% $(NH_4)_2SO_4$ saturation. F8; fraction obtained by precipitating S at pH 8 with 40–50% $(NH_4)_2SO_4$ saturation. ●—●; antidiuretic activity after incubation of vasopressin with 0·5 ml. of 0·1 M cysteine. Volumes of S, F5 and F8 were adjusted so that 0·5 ml. = 0·05 g fresh tissue.

*Inactivation of vasopressin by fraction F*8

No decrease of activity of F8 was noted after storage at −10° C for up to 4 weeks, or after dialysis for 36 hr; appreciable loss of activity, however, was observed after heating the fraction at 55° C for 15 min. The time course of the inactivation of vasopressin by F8 is illustrated in Fig. 2. While the shape of the curves was much the same whether the incubation had taken place in the presence of either oxygen or nitrogen, the rate of inactivation of vasopressin appeared to be decreased when oxygen was present. This suggested that oxygen either inactivated the enzymic system or partially reversed the process of inactivation.

It has been shown that the molecule of pure vasopressin has a disulphide link (du Vigneaud, Lawler & Popenoe, 1953) and that the antidiuretic activity of vasopressin can be abolished by cleavage of the ring at the —S—S— link (van Dyke, Chow, Greep & Rothen, 1942). As the present work was done using a commercial preparation of vasopressin, it was of importance to see whether this drug resembled pure vasopressin in so far as it had also a disulphide link. The following experiments indicate that this was likely. Ten m-u.

vasopressin in 5 ml. Krebs–Ringer solution was incubated for 10 min with 0·5 ml. of 0·1 M cysteine, of 0·002 M glutathione or of a saturated solution of cystine and then assayed. Fig. 3 shows that, with the exception of cystine, there was a decrease of the antidiuretic activity of vasopressin. The reduction in activity was immediate, and prolonging the time of incubation of vasopressin with cysteine did not lead to any further inactivation (Fig. 1). There appeared, however, to be a clear relationship between the amount of cysteine or glutathione present and the degree of inactivation of vasopressin (Table 3).

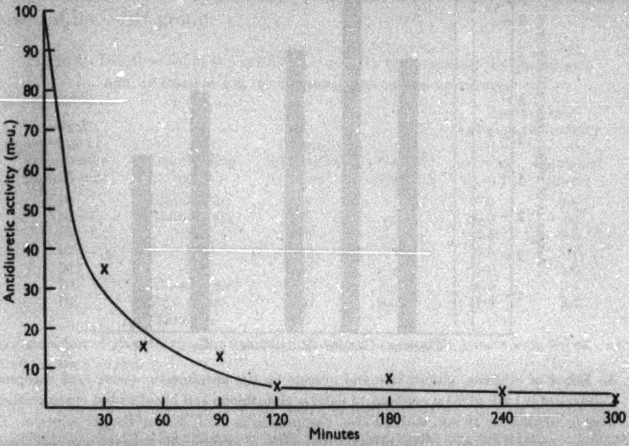

Fig. 2. Time course of inactivation of vasopressin by fraction F8, precipitated at pH 8 with $(NH_4)_2SO_4$ at 40–50% saturation. Note: the initial dose of vasopressin was 100 m-u. (instead of 10 m-u.) and the volume of F8 was 2·0 ml. = 0·2 g fresh tissue.

Reduction of the antidiuretic activity of vasopressin was also observed by addition of 0·5 ml. of $CuSO_4$ (1 mg Cu^{2+}/ml.) or of $Hg(NO_3)_2$ (1 mg Hg^{2+}/ml.) (Fig. 3).

It is of interest to note that a reduction of about one-third of the initial amount of vasopressin was achieved by using either 1 μmole glutathione or 50 μmoles of cysteine. When the concentration of glutathione was increased, much greater amounts of vasopressin were inactivated than with cysteine and it could be calculated that whereas 40 μmoles of glutathione inactivated about 30 m-u. vasopressin, 40 μmoles of cysteine inactivated about 3 m-u. vasopressin only. The difference between the abilities of these two compounds to inactivate vasopressin might be attributed to their different redox potentials. According to Ryklan & Schmidt (1944) $E^{0\prime} = +0{\cdot}15$ for cysteine and $-0{\cdot}03$ for glutathione.

The following two series of experiments indicate that the inactivation of vasopressin by glutathione may be due to a reduction of the —S—S— link of the molecule. In the first experiment two samples of vasopressin were incubated with 0·5 ml. of 0·002 M glutathione solution for 60 min. One sample was assayed without any further treatment, while the other was gassed with

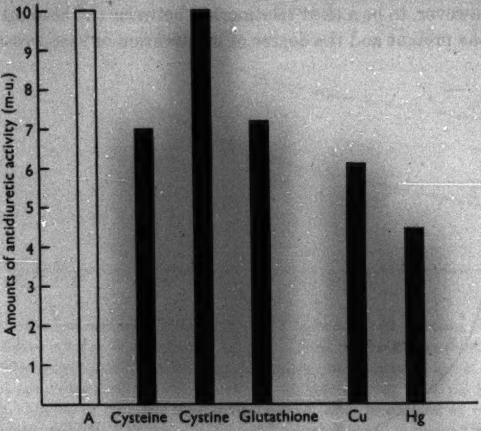

Fig. 3. Effect of cysteine, glutathione and cystine on the antidiuretic activity of vasopressin. Volumes of 0·5 ml. of 0·1 M cysteine, of 0·002 M glutathione and of saturated cystine solutions were incubated for 10 min with 10 m-u. vasopressin in 5 ml. Krebs–Ringer solution. Open column: original amount of vasopressin. Reduction of antidiuretic activity was also observed by incubating vasopressin with similar volumes of $CuSO_4$ (= 1 mg Cu^{2+}/ml.) and of $Hg(NO_3)_2$ (= 1 mg Hg^{2+}/ml.).

TABLE 3. Inactivation of vasopressin by glutathione

Initial amount of vasopressin (m-u./5 ml.)	Amount of glutathione, 0·002 M (ml.)	Time of incubation (min)	Amount of vasopressin inactivated (m-u.)
10·0	0·2	45	0·6
10·0	0·4	45	1·2
10·0	1·0	45	3·3

O_2 for 10 min before the assay. Only 80% of the activity initially present was found in the first sample, whereas in the second 100% was recovered. When 0·04 M glutathione solution was gassed with O_2 until oxidation, as shown by O_2 uptake, was complete before being incubated with vasopressin, there was no decrease of the antidiuretic activity of vasopressin (Table 4).

In the light of the results obtained with cysteine and glutathione the question arose whether the curve of inactivation of vasopressin by the fraction

F8 (Fig. 2) was due to an enzyme or simply to the cleavage of the —S—S—link by the protein-bound —SH groups of the kidney preparation. Samples of F8 were incubated with *p*-chlormercuribenzoate and the protein-bound Hg estimated. In six estimations the range of Hg^{2+} values varied from 48·5 to 85·3 μg Hg^{2+}/ml. F8, corresponding to 8·0–14 μg of —SH group. From this it could be calculated that in 0·5 ml. F8 used for the inactivation of vasopressin, the amount of protein-bound —SH was between 0·25 and 0·5 μmole, which is less than 1 % of the amount of glutathione —SH used. This made it clear that the inactivation of vasopressin by F8 could not be solely attributed to the presence of its —SH group.

TABLE 4. Inactivation of the antidiuretic activity of vasopressin by glutathione and by fraction F8, in the presence of oxygen or nitrogen

Initial amount of vasopressin (m-u./5 ml.)	Inactivating agent	Time of incubation (min)	Gas phase	Amount of antidiuretic activity	
				recovered (m-u.)	destroyed (m-u.)
10	Glutathione	60	$+N_2$	8·0	2·0
10	Glutathione	60	$+O_2$	10·0	0·0
10	F8	30	$+N_2$	3·4	6·6
10	F8	30	$+O_2$	4·5	5·5
10	F8	20	Air	5·0	5·0
10	Glutathione*	20	Air	6·6	3·4
10	F8 + glutathione*	20	Air	1·8	8·2

Concentration of glutathione was 0·002 M; amounts of inactivating agents were 0·5 ml., except for * which were 1·0 ml.

The following experiments give more information as to the mechanism of inactivation. First, the rate at which vasopressin was inactivated by F8 could be increased by addition of cysteine or glutathione (Table 3), the —SH of these compounds acting either directly on the —S—S— link of vasopressin or by maintaining the enzyme in an —SH form. Secondly, the inactivation of vasopressin by F8 was impaired by cystine; in this respect cystine acted in a way similar to that observed for F6 (Table 2). Finally, it could be shown that the amount of vasopressin inactivated by F8 could be decreased by oxygenation (Table 4). In contrast with glutathione or cysteine the reversibility of the inactivation was, however, only partial.

DISCUSSION

The incubation of vasopressin with fractions obtained from the 'particle-free' supernatant fluid of kidney homogenate has made it clear that the inactivation is a complicated process which can be achieved by several enzymes, and that some fraction exerts an inhibitory effect when incubated together with other fractions from the same kidney homogenate. In view of this unexpected complexity the present investigation was confined to the investigation of the mechanism of inactivation of vasopressin by the fraction of the supernatant fluid precipitated with the ammonium sulphate between the levels of 40 and 50 % saturation at pH 8.

As pure vasopressin contains an —S—S— link, it was reasonable to expect that this link might be broken when incubated with compounds containing an —SH group. According to van Dyke *et al.* (1942) the antidiuretic activity of vasopressin is abolished by cleavage of the ring at the —S—S— link. The drug Pitressin, used throughout the investigation, is a commercial preparation and though its makers claim for it a high degree of purity, it was necessary to ascertain whether it conformed to the structure of pure vasopressin with regard to the existence of an —S—S— bond. Reduction of the antidiuretic activity of Pitressin was observed after incubation with cysteine or glutathione, whereas incubation with cystine left its antidiuretic activity unchanged. This indicated the presence of an —S—S— link in the drug used.

There is an interesting divergence between observations made by Sealock & du Vigneaud (1935) and by van Dyke *et al.* (1942). Both groups of workers incubated Pitressin with cysteine until all the disulphide links had been transformed into the sulphydryl form. Though the drug had been entirely reduced, Sealock & du Vigneaud could not detect any loss of its *pressor* activity, while van Dyke *et al.* observed a substantial loss of the *antidiuretic* activity. The results of van Dyke *et al.* (1942) have now been confirmed. Several years ago Heller (1939) had already noted a difference between the stability of both pressor and antidiuretic activities. A difference in the metabolism of the antidiuretic and the pressor activities of Pitressin injected *in vivo* has also been postulated more recently by Dicker & Nunn (1957). In a pilot experiment, during which vasopressin had been incubated with cysteine and assayed *simultaneously* for its antidiuretic and pressor activities, it was found that while there was no decrease of the pressor activity, the antidiuretic activity was reduced by one third. This experiment, confirming both du Vigneaud's and van Dyke's findings, suggests that the responsibility for the different pharmacological activities resides with different groups of the same molecule of vasopressin. For instance, the antidiuretic activity alone would appear to be dependent on the integrity of the —S—S— link.

Chemical analysis showed that the fraction F8 from a kidney homogenate contains some sulphydryl groups. As Pitressin appeared to have an —S—S— link, it was reasonable to expect the cleavage of this link when the drug was incubated in the presence of F8. The question was then: can the cleavage of the —S—S— link be responsible for the complete inactivation of vasopressin by F8? Complete inactivation of vasopressin can be obtained by increasing the amounts of cysteine or glutathione used. Estimation of the protein-bound sulphydryl group of F8 showed, however, that whereas 4 μmole of glutathione reduced the antidiuretic activity of vasopressin by 30 %, 0·5 ml. of F8, containing less than 0·04 μmole, reduced the antidiuretic activity almost entirely to zero.

From the present experiments it can be concluded that the inactivation of the antidiuretic activity of vasopressin by the fraction F8 of a kidney homogenate is of enzymic nature, possibly acting on the —S—S— link of vasopressin, reducing it to —SH. The activation of the enzyme by —SH—containing compounds like cysteine or glutathione may indicate that the enzyme itself is an —SH enzyme with maximum activity in the —SH form.

SUMMARY

1. Crude fractionation of kidney homogenates was attempted by precipitating the 'particle-free' supernatant at various hydrogen-ion concentrations with $(NH_4)_2SO_4$ at different levels of saturation. Fractions precipitated at pH 4, 5, 7 and 8 were all able to inactivate vasopressin to some degree. The fraction precipitated at pH 8 was the most active. No inactivation was observed with the fraction precipitated at pH 6.

2. The antidiuretic activity of vasopressin is reduced by incubation with cysteine or glutathione. This inactivation is immediate and is not influenced by length of time of incubation, but can be reversed by oxygenation. The degree of inactivation appears to be related to the amount of —SH groups added.

3. The time course of inactivation of vasopressin by the fraction of kidney homogenate precipitated at pH 8, between the levels of 40 and 50% $(NH_4)_2SO_4$ saturation, resembles that of an enzymic reaction: the inactivation can be reversed only partly by oxygenation.

4. Almost complete inactivation of vasopressin (10 m-u.) was observed with 0·5 ml. of fraction F8, containing between 8 and 14 μg of —SH group, which is less than 1% of the glutathione —SH which can inactivate about 3·0 m-u. only.

5. The inactivation of the antidiuretic activity of vasopressin by fraction F8 may be due to an enzyme acting on the —S—S— link of the drug, reducing it to —SH; it is suggested that the enzyme is an —SH enzyme with a maximum activity in the —SH form.

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THE DEPRESSION OF THE RECEPTOR POTENTIAL IN PACINIAN CORPUSCLES

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Gray & Sato (1953) showed that when two successive receptor potentials are generated in a Pacinian corpuscle the second is diminished. This depression increased as the first, or conditioning, stimulus was increased and also as the interval between the two responses was reduced. The observations of Gray & Sato were made on preparations in which nervous impulse activity had been abolished. It is possible, however, to observe depression after an impulse. This paper extends the observations of Gray and Sato and includes others on preparations in which impulse activity was not abolished, the conditioning response being either an impulse arising from a receptor potential or an antidromically conducted impulse.

The experiments described, as well as giving further information about the relation of depression to the conditioning response, had a second object. Diamond, Gray & Sato (1956) produced evidence that, after mechanical stimulation, all-or-nothing activity does not occur in the non-myelinated terminal but starts at the first node of Ranvier. A comparison of the depression caused by mechanically excited impulses with that caused by antidromically conducted ones has throw some light on the question of whether or not an antidromically conducted impulse invades the non-myelinated terminal.

METHODS

Single Pacinian corpuscles and their axons were dissected as described by Gray & Sato (1953) and mounted in the manner of Diamond *et al.* (1956), the solution used being a modification of Krebs-Henseleit's solution having the following composition (mM): NaCl 145, $CaCl_2$ 2·5, $MgSO_4$ 1·2, KH_2PO_4 0·58, K_2HPO_4 2·5, HCl 0·3. The mesenteric nerve which included the axon supplying the corpuscle was mounted on platinum electrodes so that impulses might be excited which would be conducted to the corpuscle antidromically (Gray & Sato, 1953).

The stimulator with its photo-electric recording device was that described by Gray & Malcolm (1951).

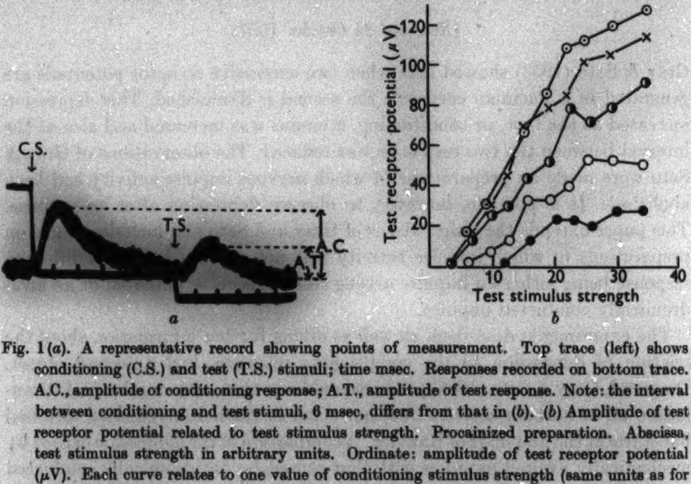
The recording technique was as previously described (Gray & Sato, 1953; Diamond *et al.* 1956). A differentiating circuit was used to measure the rate of rise of the receptor potential in one experiment. The circuit used had a time constant of 16 μsec and was calibrated by means of linearly increasing potentials.

Each record from which measurements were made consisted of a number of superimposed traces, usually 25, except in one or two experiments in which the records were of single traces.

RESULTS

Depression after receptor potentials alone

A number of experiments were done in which the effects of one receptor potential on a succeeding one were observed in the absence of impulse activity, which was abolished with procaine. The results described in this section are largely drawn from one experiment which was typical of all experiments, was the most complete and gave the most consistent results. In this experiment the strengths of the first (conditioning) and the second (test) mechanical stimuli were varied in the following manner. For each value of the conditioning stimulus ten test stimuli were given, these being such that the receptor potentials resulting from them, in the unconditioned state, ranged from zero to about 90% of maximum. Ten values of conditioning stimulus were used ranging from zero to the largest available. A complete run therefore consisted of 10 different test stimuli for each of 10 different conditioning stimuli; such a run was performed for each of a number of time intervals between the conditioning and test stimuli. In Fig. 1*b* are shown curves, each of which shows the relationship between the test response and the test stimulus for one strength



a *b*

Fig. 1 (*a*). A representative record showing points of measurement. Top trace (left) shows conditioning (C.S.) and test (T.S.) stimuli; time msec. Responses recorded on bottom trace. A.C., amplitude of conditioning response; A.T., amplitude of test response. Note: the interval between conditioning and test stimuli, 6 msec, differs from that in (*b*). (*b*) Amplitude of test receptor potential related to test stimulus strength. Procainized preparation. Abscissa, test stimulus strength in arbitrary units. Ordinate: amplitude of test receptor potential (μV). Each curve relates to one value of conditioning stimulus strength (same units as for test stimulus) as follows: ⊙, unconditioned; ×, 11; ◑, 18; ○, 26; ●, 75: interval between conditioning and test stimuli 2 msec. Same preparation as Figs. 2–4.

of the conditioning stimulus. Five of the ten values of conditioning stimulus strength used are represented. All these curves were obtained at a time interval between conditioning and test stimuli of 2 msec. Complete series of this type were obtained at the following intervals and in the following order 3, 5, 2, 4, 1, 3, 7, 6, 9, 3 msec. In some other experiments longer time intervals were used. The stability of the preparation throughout the experiment was indicated by the similarity of the different curves obtained for the 3 msec interval and the constancy of the responses to a given conditioning stimulus; the standard deviations of the observations ranged from 2·3 to 3·5 μV.

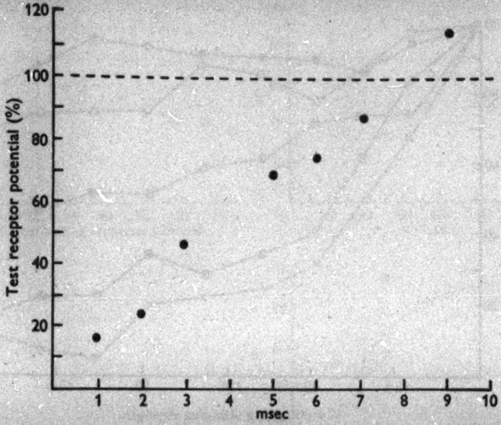


Fig. 2. Time course of depression. Procainized preparation. Abscissa: interval between conditioning and test stimuli in msec. Ordinate: amplitude of test receptor potential as percentage of the unconditioned value. Means of runs at four values of test stimulus. Conditioning stimulus strength 51 u. (see Fig. 4*d*). The dotted horizontal line is at 100% on the ordinate. Same preparation as Figs. 1, 3, and 4.

From Fig. 1*b* it can be seen that a conditioning receptor potential reduced the response to the test stimulus at all values of test stimulus strength used. The amplitude of the response to a particular test stimulus can best be expressed as a percentage of the response to the same test stimulus in the absence of any conditioning activity, and this is a measure of the depression. When this was done for the results of the experiment illustrated in Fig. 1*b* it was found that the test stimulus strength had little or no influence on the depression. In two other series, however, the depression was greater when the test stimulus was small.

The degree of depression depends on the interval between conditioning and test stimuli. Gray & Sato (1953) observed depression for 20–25 msec after the conditioning stimulus was applied; in the present experiments, however, the maximum time interval at which depression was detectable was 10 msec. The curve shown in Fig. 2 was obtained by calculating the percentage responses to four different values of test stimulus and plotting the mean against the time interval; all points were obtained with a conditioning stimulus strength of 51 units (see Fig. 4*d*). This result is typical of most experiments, though the increased response observed at 9 msec was not a consistent finding.

The relationship between conditioning activity and depression is shown in Fig. 3. In this figure the percentage response is plotted against conditioning stimulus strength; all these results were obtained with the same test stimulus strength but the time interval between conditioning and test stimuli was varied; each curve refers to a different time interval. Other series at other test stimulus strengths were similar to that illustrated, as would be expected from what has been said above. The family of curves in Fig. 3, which is typical, shows that depression increased at first rapidly and then more slowly as the conditioning stimulus strength was increased. The curves representing different intervals have essentially the same shape, and it therefore seemed justifiable to analyse in detail the results obtained at one interval. The interval chosen was 3 msec, at which more than one series of observations was made; the mean of the values of depression at four test stimulus strengths from two such series is plotted in Fig. 4*a* against the conditioning stimulus strength.

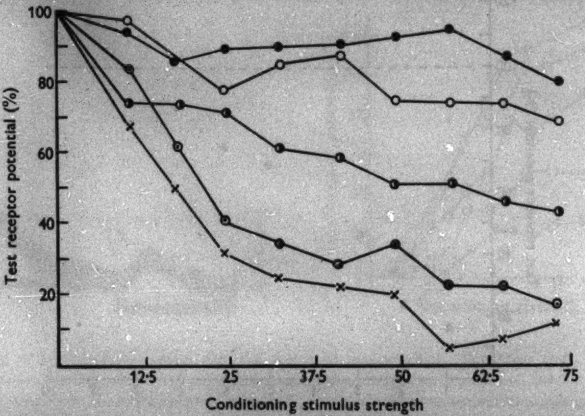


Fig. 3. Depression of test receptor potential related to conditioning stimulus strength. Procainized preparation. Abscissa: conditioning stimulus strength in arbitrary units. Ordinate: amplitude of test receptor potential as percentage of the unconditioned value. Each curve relates to one interval in msec between the conditioning and test stimuli as follows: ×, 1; ⊙, 2; ◑, 4; ○, 6; ●, 7. Test stimulus strength 30 u. throughout. Same preparation as Figs. 1, 2 and 4.

This and other such curves show that depression continued to increase up to the limit of conditioning stimulus strength used. This is not true, however, of the amplitude of the conditioning response, which reached a maximum value before the limit of conditioning stimulus was reached. This is shown in Fig. 4*d*, all the values plotted being means of eight observations. It is clear that changes of conditioning stimulus strength produced considerable changes in depression at a level at which they produced little change in the amplitude of the receptor potential. This is more clearly shown in Fig. 4*c*, in which the actual values of depression are plotted against the amplitude of the conditioning response. The slope of the curve becomes much steeper as the amplitude of the conditioning response increases; in at least one other experiment the line became vertical, though the limits of error are such that the results are consistent with those shown in Fig. 4*c*. In view of these results we have also considered the relationship between the rate of rise of the conditioning response

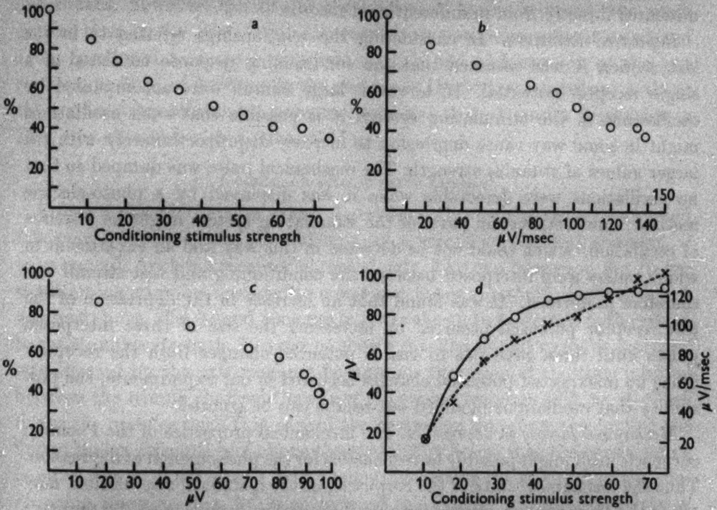


Fig. 4. Depression of test receptor potential related to parameters of conditioning receptor potential. Procainized preparation. Abscissae: *a* and *d*, conditioning stimulus strength in arbitrary units; *b*, maximum rate of rise of conditioning receptor potential (μV/msec); *c*, amplitude of conditioning receptor potential (μV). Ordinates: *a*, *b* and *c* amplitude of test receptor potential as percentage of the unconditioned value (mean values resulting from four test stimulus strengths in each of two series of observations); *d* (left), amplitude of conditioning receptor potential (μV), circles; (right) maximum rate of rise of conditioning receptor potential (μV/msec), crosses. Values of amplitude plotted in *d* are means of eight observations and have a S.E. of 0·9–1·3 μV. Interval between conditioning and test stimuli 3 msec. Same preparation as Figs. 1–3.

and the depression, since it has already been shown (Gray & Sato, 1953) that even when the receptor potential has reached its maximum amplitude its rate of rise still increases with increasing stimulus strength. Fig. 4*d* shows the relationship between stimulus strength and rate of rise of receptor potential for the preparation from which the previous results were obtained. The values of rate of rise plotted were obtained from a differentiated record and thus represent the maximum rate of rise. Fig. 4*b* is a graph relating depression and the rate of rise of the conditioning response. This result is similar to those obtained in other experiments in which rate of rise of receptor potential was measured directly from undifferentiated records.

Repetitive activation. In considering the relationships referred to in the last section it was assumed that the conditioning response consisted of a single receptor potential. If, however, large stimuli were accompanied by oscillations in the stimulating system it is possible that such oscillations might in some way cause depression to increase disproportionately with the larger values of stimulus strength. The mechanical pulse was damped so that no oscillations were detectable when it was displayed by a photo-electric method; however, certain parts of the stimulating system might be a source of oscillations which could not be detected in this way and an experiment in which pulses were interposed between the conditioning and test stimuli was therefore performed. It was found that no increase in the depression of the test receptor potential occurred on increasing the size of three interposed pulses until these gave rise to visible potential changes from the receptor. Since no unexpected potential changes occurred in our experiments, the possibility that oscillations modified our results can be ignored.

Mechanical factors in depression. The mechanical properties of the Pacinian corpuscle itself might possibly be responsible for the phenomenon of depression. Thus the mechanical state of the corpuscle, at the instant of stimulation, may affect the transmission of the stimulus through the corpuscle to the sensitive element. This seems improbable since we have observed the same pattern of depression when using the following as conditioning and test stimuli: two pulses, two steps of compression or two steps of decompression (examples are shown in Figs. 1*a* and 5). Hubbard (1958) has shown that the mechanical response of a corpuscle to a maintained compression consists of a static and of a dynamic component. Only the dynamic component can be observed near the core of the corpuscle and appears to be the immediate precursor of the receptor potential. The experiments we have described above do not exclude the possibility that interaction of the dynamic components cause depression.

That depression is dependent on the electrical responses is shown by the experiment using repetitive activation (above). In one part of this experiment three short identical pulses preceded a test stimulus by 3, 2 and 1 msec, and the effect of changing the size of these pulses was observed. Pulses smaller than a certain value had no detectable effect; when their size was increased beyond this value, however, a receptor potential evoked by the pulses was observed, together with depression of the test response. The relationships between these two effects and size of pulse had the typical forms (see Fig. 4*a*, *d*). In the second part of the experiment a conditioning stimulus, which was identical with the test stimulus, preceded the first of the pulses by 2 msec. This extra stimulus did not, by itself, depress the test response. Depression of the test response again appeared when the receptor potentials due to the interposed pulses reached the critical value referred to above; in this part of the experiment, however, the size of the interposed pulses required was much greater than in the other. With the conditioning stimulus present a pulse of 116 u. produced a receptor potential of $9\mu V$ and a depression of the test response to 78% of its value in the absence of the interposed pulses; without the conditioning stimulus a receptor potential of $9\,\mu V$ and a depression to 78% was obtained with a pulse of 35 u.

Depression after a nerve impulse

Evidence that the impulse causes depression. During the refractory period following a mechanically excited impulse a second mechanical stimulus, if large enough, evokes a receptor potential only. This can be seen in Fig. 5; the conditioning stimulus recorded in *a* was just below threshold, while that in *b* was just above. The second response in *b* was shown to be graded with respect to the stimulus strength. It could be shown that the second receptor potential was affected by the first response because an alteration of the time interval between the stimuli changed the size of the second response (Fig. 5*c*, *d*). That it was the impulse itself that caused the depression was shown by setting the first stimulus strength just above threshold (Fig. 5*e*) so that the conditioning impulse was initiated after a long latency; increasing the strength of the conditioning stimulus (Fig. 5*f*) then reduced this latency of initiation (Gray & Malcolm, 1950) and in consequence increased the interval between the conditioning impulse and the test receptor potential. This caused an increase in the second response in spite of the increase in conditioning stimulus strength which, in the absence of an impulse, would have increased the depression (Figs. 3, 4*a*). The amplitude of the second response has been plotted against the interval between the conditioning impulse and the test receptor potential, both when the interval was altered by altering the time of application of the test stimulus, and also by changing the strength of the conditioning stimulus. The two curves are plotted in Fig. 6 and are very similar; if the depression were due to the first receptor potential the curves would be expected to slope in opposite directions.

If the strength of the conditioning stimulus, in a pair such as those shown in Fig. 5, was increased steadily from zero to the maximum available and the

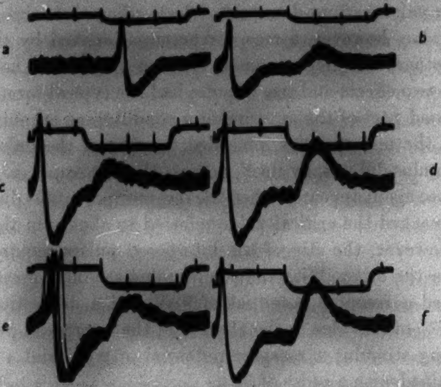


Fig. 5. Depression of a receptor potential after an impulse. Non-procainized preparation. Top beam signals conditioning and test stimuli and time 1 msec in each record. Responses recorded on bottom beam. *a*, *b*: conditioning stimulus in *a* just below threshold, in *b* just above. *c*, *d*: effect of changing the conditioning–test stimulus interval on a test receptor potential elicited as in *b*. Note: strength of conditioning and test stimuli in *c* identical with those in *d*. *e*, *f*: effect of changing latency of initiation of conditioning impulse on a test receptor potential elicited as in *b*. Note: conditioning–test stimulus interval in *e* identical with that in *f*. All records are from the same preparation. Amplification of top beam same throughout, that of bottom beam higher for *c*–*f* than for *a* and *b*. Conditioning stimulus applied at beginning of sweep in each record.

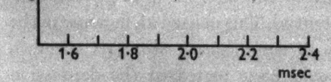


Fig. 6

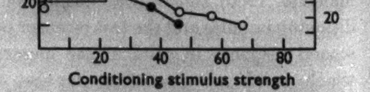


Fig. 7

Fig. 6. Amplitude of test receptor potential related to interval between conditioning impulse and test response. Non-procainized preparation. Abscissa: interval between conditioning impulse and test receptor potential (msec) as produced by: ○, varying stimulus interval; ●, latency change of conditioning impulse. Ordinate: amplitude of test receptor potential (μV).

Fig. 7. Amplitude and rate of rise of test response related to conditioning stimulus strength. Non-procainized preparation. Abscissa: conditioning stimulus strength in arbitrary units. Ordinates: (left) amplitude of test response (μV), open circles; (right) rate of rise of test response (μV/msec), closed circles. Interval between conditioning and test stimuli 3 msec. Test stimulus strength 7 u. The test response included an impulse at the point indicated by a cross. Inset: small rectangle × 2.

amplitude of the response to a standard test stimulus plotted against it, then a curve such as that shown in Fig. 7 (open circles) was obtained. The first point on the left is indicated by a cross showing that the test response included an impulse. An increase in the strength of the conditioning stimulus then brought in the conditioning impulse and the test response fell to a low level. Further increase in the conditioning stimulus strength caused an increase in the test response as the latency of the conditioning impulse decreased, until a maximum was reached. As the stimulus strength increased further the amplitude of the test response declined. Changes in the rate of rise of the test receptor potential with changing conditioning stimulus strength ran parallel with the changes in amplitude (Fig. 7, closed circles). In one experiment the depression caused by the largest available conditioning stimulus with the impulse present was compared, over a small range of intervals and a wide range of test stimulus strengths, with the depression caused in the same preparation by the same conditioning stimulus after the impulse had been abolished with procaine; there was good agreement at all points. In an experiment such as that illustrated in Fig. 7, in which an impulse occurs in response to the conditioning stimulus, the recorded response to this stimulus remains the same at all suprathreshold values; under the conditions of recording it is the all-or-nothing activity of the second node of Ranvier that is being seen and all activity peripheral to this node becomes invisible (Diamond *et al.* 1956). However, we assume that the activity responsible for the receptor potential is increasing with the increasing stimulus strength. The depression caused at various conditioning stimulus strengths by a pure receptor potential (Figs. 3, 4*a*) can be compared with that caused by a receptor potential plus an impulse (Fig. 7). It appears that the all-or-nothing impulse causes a depression of the receptor potential that is greater than that caused by a just subthreshold receptor potential, but that with larger stimulus strengths the increasing depression is caused by increasing receptor activity.

Comparison of depression caused by mechanically excited and antidromically conducted impulses. Fig. 8 illustrates the result of a typical experiment in which depression due to an impulse excited by mechanical stimulation of the receptor was compared with that following an impulse conducted antidromically. The dotted lines in this figure show the relationship between the receptor potentials following mechanical test stimuli and the time interval between conditioning impulse and the test stimulus; the four parts of the figure correspond to four values of test stimulus. It was impossible in the experiment to use intervals longer than those indicated in the figure because the receptor potentials were then completely obscured by impulses; even certain of the points plotted are liable to some inaccuracy because impulses were superimposed on the receptor potentials. 7 msec was an unusually long time after the conditioning impulse for there to be a test response consisting of a pure receptor potential, and in most experiments 4–5 msec was the longest interval.

Antidromically conducted conditioning impulses were set up by stimulation of the appropriate mesenteric nerve. The receptor was then tested in the manner already described by using mechanical test impulses of varying strength at various intervals after the arrival of the conditioning impulse. A family of curves, comparable with those found after a mechanical conditioning stimulus, was thus obtained (Fig. 8, full lines), and to facilitate the comparison both experiments were done on the same preparation in the following way. Four series of observations were made, in the first and last of which mechanical conditioning stimuli were used, while in the second and third the conditioning impulses were conducted antidromically. The latencies between the beginning of the conditioning stimulus and the beginning of impulse activity peripheral

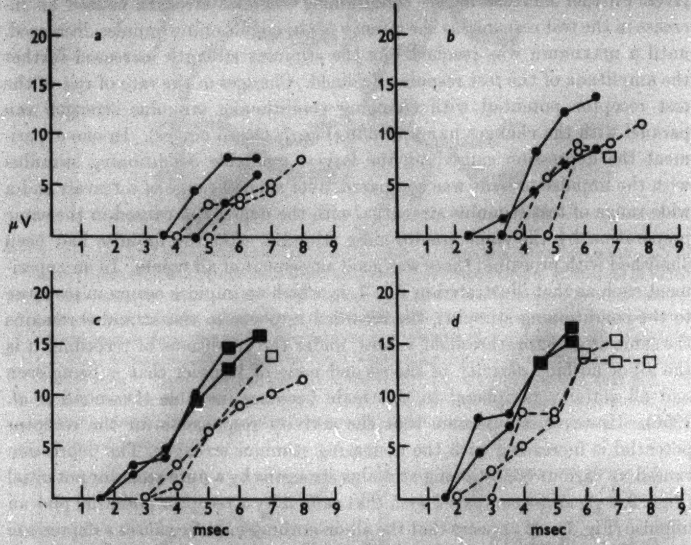


Fig. 8. Amplitude of test receptor potential related to interval between conditioning impulse and test response. Non-procainized preparation. The four parts of the figure correspond to four values of test stimulus (arbitrary units) as follows: *a*, 19; *b*, 26; *c*, 32; *d*, 38. Abscissae: interval between conditioning stimulus and test response (msec). Ordinates: amplitude of test receptor potential (μV). Open symbols, dotted lines: conditioning impulse excited mechanically. Closed symbols, solid lines: conditioning impulse conducted antidromically. Each of the four curves in each part of the figure corresponds to one series of observations (see text). Square symbols indicate that an impulse was superimposed on the test receptor potential, the amplitude of which was estimated.

to the recording barrier were then measured with a faster oscillograph sweep, and subtracted from the interval between conditioning and test stimuli; the latency of the mechanically excited impulse was taken to the first sign of the impulse and that of the antidromically conducted impulse to the beginning of the downstroke of the diphasic action potential (Fig. 9). In Fig. 8 four sets of such observations have been plotted; each part of the figure represents a particular value of test stimulus strength and in each part are four curves corresponding to the four series of observations referred to above. It will be seen that there is good agreement between each of the pairs of curves but that there is a difference between those conditioned mechanically and those conditioned antidromically. At all corresponding times and at all corresponding stimulus strengths in all preparations the depression caused by the antidromically conducted impulse was less than that caused by the mechanically excited impulse.

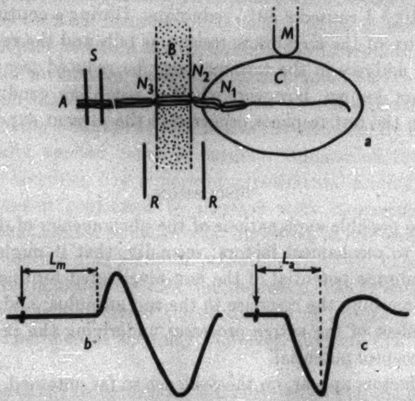


Fig. 9. Diagram illustrating measurement of latency of conditioning nerve impulses. *a*: *C*, Pacinian corpuscle; *A*, axon; N_1, N_2, N_3, nodes of Ranvier; *B*, petroleum jelly barrier; *R*, recording electrodes; *S*, stimulating electrodes; *M*, mechanical stimulator. *b* and *c*: stimulus applied at arrow; *b*, mechanically excited impulse, latency, L_m; *c*, antidromically conducted impulse, latency, L_a.

This might be explained by the depressing effect of the receptor potential which always accompanies the mechanically excited impulse. From a series of curves such as that in Fig. 7 (open circles) a conditioning mechanical stimulus was found which caused minimal depression and excited an impulse. Even when this conditioning stimulus was used it caused more depression than that which followed an antidromic impulse.

Depression due to applied currents

In view of the possible association between depression and depolarization of the terminal membrane it was considered advisable to investigate the effects of externally applied currents. A short current pulse, 2 msec in duration, was used and the test receptor potential was excited 3 msec after the end of this pulse. It was found that following cathodal current pulses greater than 5×10^{-7} A the recorded receptor potential was reduced; increasing the current pulses to 18×10^{-7} A caused a 50% reduction. During a continuously applied depolarizing current the membrane resistance falls and the recorded currents resulting from activity in the terminal may be reduced (see Diamond *et al.* 1956). It is not known, however, whether recording conditions were still disturbed when the test response occurred in the present experiments.

DISCUSSION

There are three possible explanations of the phenomenon of depression: first, that it is due to mechanical factors; secondly, that it might result from a change of membrane potential of the non-myelinated terminal in the period immediately preceding the response to the test stimulus; and, thirdly, that it might be an effect of the active processes underlying the production of the conditioning receptor potential.

Mechanical factors appear, on the evidence so far obtained, to play little or no part in the production of depression: compressions, decompressions and short pulses all produce essentially the same results, and the experiment with interposed pulses suggests that depression depends on the conditioning response rather than the conditioning stimulus. It would be interesting to know what mechanical interaction can take place in the dynamic component of the radial displacement measured near the central core (Hubbard, 1958).

The explanation that at first seems most satisfactory is that depression is the result of a potential change occurring across the membrane of the non-myelinated terminal. Diamond *et al.* (1956) have argued that a mechanically excited pulse is initiated at the first node of Ranvier, and it seems reasonable to assume (Gray, 1956) that the receptor potential is developed across the membrane of the non-myelinated terminal. If these assumptions are true it is difficult to explain how an impulse can depress a receptor potential unless the depression is due to the change of membrane potential produced in the non-myelinated terminal by current resulting from the activity at the first node of Ranvier. The finding that the depression that occurs after large conditioning stimuli is greater than that caused by a conditioning impulse (Fig. 7) could also be explained in terms of potential change in the terminal. It has been shown that the receptor potential is of the same order of magnitude as the action and resting potentials (Diamond *et al.* 1956); the potential change across the non-myelinated terminal membrane due to an impulse at the first node might well, as a result of attenuation across the first internode, be less than the potential change due to a maximum receptor potential. The attenuation across this first internode is likely to be quite large because the over-all impedance across the membrane of the non-myelinated terminal may be expected to be relatively low; this attenuation may be greater during receptor potential activity which may reduce the impedance still further. If the potential change across the terminal membrane due to an impulse is less than that due to a maximal receptor potential and the cause of the depression is the potential change across this membrane, then large stimuli would be expected to cause greater depression than small ones even in the presence of an impulse. The explanation invoking a change of potential is not excluded by the experiment with an externally applied cathodal current. Against all these arguments must be placed, however, one important experimental finding. In some experiments depression continued to increase with increasing conditioning stimuli beyond that needed to evoke what appeared, at our level of resolution, to be maximal conditioning receptor potentials (Fig. 4). This finding must throw considerable doubt on the idea that depression can be entirely explained by a change of potential across the membrane of the axon terminal; the limitations of experimental accuracy, however, prevent this evidence from being conclusive.

The rate of rise of the receptor potential must depend on the current flowing into the membrane capacity. This current may be expected to reflect an underlying process, say a change in membrane permeability to one or more ion species (Gray, 1956; Diamond, Gray & Inman, 1957). Fig. 4*d* shows that depression and the rate of rise of the conditioning receptor potential are related, but there is no definite evidence that this relationship is causal. Moreover, an explanation involving the underlying processes does not explain depression by mechanically excited impulses unless the evidence (Diamond *et al.* 1956; Lowenstein & Rathkamp, 1957) that such impulses are initiated at the first node of Ranvier is rejected.

At the present time no final conclusion can be reached about the mechanism of depression. Perhaps the simplest explanation is that more than one process is involved and that either a change of membrane potential or the active process can modify the production of a subsequent receptor potential.

A mechanically excited impulse can cause depression of a receptor potential and there are good reasons for thinking that such impulses are initiated at the first node of Ranvier (Diamond *et al.* 1956; Lowenstein & Rathkamp, 1957). If an antidromically conducted impulse invaded the non-myelinated terminal it would produce a larger change of membrane potential there than would the attenuated mechanically excited impulse; it would also cause all-or-nothing activity of the terminal membrane itself. Appreciably more depression would be expected to result from such an antidromic invasion than from an impulse at the first node. Experimental findings show that the depression resulting from an antidromically conducted impulse is no greater than that due to an impulse excited mechanically. If the evidence that the latter is initiated at the first node is accepted then it must follow that the antidromically conducted impulse does not invade the terminal.

This conclusion is similar to that reached by Eyzaguirre & Kuffler (1955*b*). They concluded that antidromically conducted impulses do not invade the terminals of the stretch receptor of the crayfish. In this receptor (Eyzaguirre & Kuffler, 1955*a*) and in the Pacinian corpuscle there is therefore evidence that impulses, whether excited mechanically or antidromically, do not invade that part of the nerve fibre which is involved in the receptor activity. It is possible that these terminals are unable to conduct all-or-nothing impulses; this reinforces the evidence that the terminal is highly specialized for the production of receptor potentials and may have quite different properties from the rest of the axon.

SUMMARY

1. The depression by a preceding stimulus of the receptor potential resulting from a mechanical stimulus has been studied in isolated mesenteric Pacinian corpuscles of the cat.

2. This depression, in corpuscles treated with procaine to abolish nervous impulses, has been related to the amplitude and rate of rise of the conditioning receptor potential and to the interval between conditioning and test stimuli.

3. Experiments have been done which indicate that depression is unlikely to be due to mechanical factors.

4. In the non-procainized corpuscle both mechanically excited and antidromically conducted nervous impulses have been shown to cause depression of a subsequent receptor potential. Increased depression results from the response to a large mechanical stimulus. This increase can be accounted for by the contribution of the receptor potential which accompanies the impulse.

5. Antidromically conducted impulses have never caused more depression than mechanically excited ones.

6. It is concluded that a change of membrane potential in the non-myelinated terminal can cause depression; this may not, however, be a complete explanation.

7. It is also concluded that antidromically conducted impulses do not invade the non-myelinated terminal.

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THE ACTION OF RESERPINE ON THE PERIPHERAL SYMPATHETIC SYSTEM

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Reserpine causes the release of 5-hydroxytryptamine (5-HT) from all tissues in which it is normally stored. This release is shown by a fall in the tissue concentration of 5-HT and by an increase in its urinary metabolites (Pletscher, Shore & Brodie, 1955; Shore, Silver & Brodie, 1955). That reserpine may also lower the concentration of catecholamines in tissues was shown by experiments (Holzbauer & Vogt, 1956) in which the disappearance of noradrenaline from the hypothalamus was demonstrated in cats injected with small doses of reserpine (0·4 mg/kg). This depletion, however, did not occur in all organs, the denervated, in contrast to the innervated, adrenal medulla remaining unaffected by this dose of the drug.

It is tempting to try to correlate the effects on behaviour and responsiveness produced by reserpine with the loss of 5-HT and noradrenaline from the brain. The tendency has been to attribute not only the sedation, but also such signs as the fall in blood pressure, the miosis and the relaxation of the nictitating membrane to central changes alone. Although all these phenomena might be due to central causes, they could also be produced, at least partly, by disturbances in the peripheral sympathetic system. Failure of another sympathetic mechanism after reserpine was described by G. M. Everett who observed (personal communication, and Everett, Toman & Smith, 1957) that cold did not produce pilo-erection in mice injected with reserpine. This failure, too, might be due to a central or to a peripheral derangement, or to both.

The aim of the present paper is to determine the effect of reserpine on the peripheral sympathetic tissues: the investigation, abstracts of which have been published (Muscholl & Vogt, 1957*a*, *b*), deals with the loss of transmitter and the damage to function produced by reserpine in adrenergic neurones. In preliminary experiments the normal range of concentrations of adrenaline and noradrenaline in various parts of the sympathetic system was established.

METHODS

The experiments were done on adult rabbits, cats and dogs.

Operative procedures

Rabbits. The left superior cervical ganglion was removed aseptically under ether anaesthesia for subsequent estimation of its catecholamines. The wound was sewn up, and the animal recovered in a few minutes. It was then given reserpine, either intraperitoneally or into the marginal ear vein. After a varying time interval the rabbit was killed by a blow on the neck and bled out, and the right superior cervical ganglion and other tissues were removed for analysis for adrenaline and noradrenaline. When the right cervical sympathetic was stimulated, the rabbit was subjected to a brief ether anaesthesia before being killed, the cervical sympathetic chain was cut and the fibres were stimulated pre- and post-ganglionically, distal to the cut. The threshold voltage was determined which produced responses of eyelid and pupil. Stimulation of the hypogastric nerves was, at first, also done under ether anaesthesia; later it was found that the same thresholds were obtained when the animal was quickly killed by exsanguination and stimulation was carried out immediately. The best responses were seen on the vas deferens and the prostate. A Ritchie–Sneath stimulator supplied square pulses of 10 msec duration at a frequency of 33 c/s. A resistance of 0·3 MΩ was placed in series with the tissue; this reduced the current supplied to a suitable level and made the effective current in different animals less dependent on tissue resistance.

For all nervous tissue other than the superior cervical ganglia control figures were obtained on normal rabbits killed by a blow on the neck. These figures were then compared with those from animals injected with reserpine. This type of comparison was, of necessity, more statistical in character than that in which the right and left ganglia of the same animal were compared. A single superior cervical ganglion was often too small for the estimation of *adrenaline*, and in a number of experiments ganglia of several rabbits were pooled.

Cats. Essentially the same procedures were used in cats. The left superior cervical ganglion was removed in an aseptic operation under ether, and reserpine injected; on the next day the cat was given ether if electrical stimulation of the right sympathetic chain was required. After the stimulation the cat was killed by cutting the vessels in the neck. When stimulation of nerves was not intended, or when it was essential to avoid activation of the adrenal medulla by the anaesthetic, the cat was rapidly exsanguinated under chloroform.

For dissection of the splenic nerves the spleen was excised with the whole length of the splenic and coeliac arteries and a small segment of attached aorta, and the fat was congealed by keeping the tissue for at least half an hour at 4° C. Most of the mesenteric fat could then be lifted off, and the nerves dissected along the artery. Between 35 and 45 mg of tissue were usually obtained; the contamination with fat and connective tissue must have been about the same in all cats, since the concentration of amines in normal controls varied but little.

Decentralization of the left superior cervical ganglion was effected by excising about 1 cm of the preganglionic sympathetic chain, the procedure being carried out aseptically under ether.

Another preliminary operation required in some experiments was unilateral adrenal denervation. Under ether anaesthesia and with aseptic precautions, the splanchnic nerves were cut and the upper three lumbar ganglia excised on the left side. There were no complications from any of these operations.

The injections of reserpine were made intraperitoneally or intravenously. In excitable cats the intravenous injections were made under chloroform.

Dogs. The hypogastric nerves were stimulated under ether anaesthesia.

Estimation of amines

The freshly excised tissues were rapidly weighed and immersed in 1 ml. acid ethanol (0·1 ml. conc. HCl: 100 ml. ethanol) cooled in a mixture of CO_2 and acetone. The tissue was homogenized and extracted in acid ethanol, the extract purified, chromatographed on paper, and the regions containing the individual amines were eluted separately. The amount of amines in the eluates was determined by bioassay, the blood pressure of the pithed rat (Shipley & Tilden, 1947) being used for noradrenaline and for amounts of adrenaline exceeding 5–10 ng (total), the rat's uterus stimulated by carbachol for smaller quantities of adrenaline and for isoprenaline. The latter amine was assayed simultaneously on the rat's auricles (Garb, Penna & Ganz, 1956). The threshold dose which produced an inotropic effect was usually 50–100 pg (10^{-12} g) D,L-isoprenaline added to a 6 ml. bath. The threshold on the rat's uterus was 50–100 pg in a 2 ml. bath. In view of the very small quantities of amine which exert effects on these organs, it is essential to avoid washing the region of the chromatograms carrying the control spots in the same benzene as the part of the paper to be eluted; otherwise there may be contamination of the paper with traces of isoprenaline coming off the control spots.

The ratio of the dose of isoprenaline to the equivalent dose of adrenaline varied from 0·1 to 0·25 on the uterus and from 0·02 to 0·07 on the auricles.

The pithed rat keeps its sensitivity to the smallest doses of noradrenaline much longer than the hexamethonium-treated rat previously used. It has the further advantage of a high sensitivity to adrenaline; it will often respond to as little as 1 ng. Details of the methods of extraction and assay, of necessary precautions and of recoveries of adrenaline and noradrenaline have been published (Vogt, 1952, 1953, 1954). Recoveries of isoprenaline (10 ng) were the same as those of adrenaline.

RESULTS

Noradrenaline and adrenaline: normal concentrations and effect of a single dose of reserpin

Paravertebral sympathetic system

Superior cervical ganglion

Rabbits. The noradrenaline concentration in the superior cervical ganglia of 41 normal rabbits was found to be 4·5 ± 0·26 μg/g (mean ± s.e. of the mean, here and throughout the paper). Table 1 shows that, in 21 of 22 experiments,

TABLE 1. Effect of injections of reserpine on the noradrenaline concentrations (μg/g fresh tissue) in the superior cervical ganglia of rabbits. (Left ganglion before, right after reserpine)

Intraperitoneal					Intravenous			
		Noradrenaline					Noradrenaline	
Rabbit no.	Dose of reserpine (mg/kg)	Left side (μg/g)	Right side (μg/g)	Loss (% of left side)	Rabbit no.	Dose of reserpine (mg/kg)	Right side (μg/g)	Loss (% of normal mean)
1	0·18	5·2	4·7	10	18	0·1	0·8	82
2	0·2	4·4	2·0	55	19	0·1	3·1	31
3	0·4	4·4	1·9	56	20	0·2	1·2	73
4	0·8	3·3	<0·6	>81	21	0·2	2·2	51
5–7	1·4	5·1	1·5	70	22	1·0	0·5	81
8–10	1·6	4·0	2·0	50	23	1·0	0·4	91
11	1·6	3·7	2·3	39	24	1·2	1·45*	68
12	1·6	4·4	1·3	70	25	1·2	1·35*	70
13	1·9	6·6	1·6	76	26–28	1·6	0·3	93
14–16	2·1	5·0	<0·4	>92	29	5·0†	0·35*	92
17	2·3	7·0	0·7	90				

The time interval between injection and removal of the right ganglion varied from 14 to 24 hr.

* Both ganglia extirpated together at the end of the experiment.

† Iproniazid, 100 mg/kg i.v., 1 hr previous to reserpine.

the noradrenaline content of cervical ganglia extirpated on the day following one injection of reserpine was much lower than normal. The only exception was rabbit 1, injected with the smallest intraperitoneal dose; even here the concentration after reserpine was 10% lower than that before. Generally speaking, the loss increased with the dose up to 0·8 mg/kg, and was greater when the same dose had been given intravenously than when it had been injected intraperitoneally. There were, however, large individual variations, as was also evident from the variable degree of sedation achieved.

In order to ascertain that the loss of noradrenaline was due to the drug and not to other factors, five rabbits were given either no injection or the reserpine vehicle only, and their left and right superior cervical ganglia were analysed; the ganglia were obtained by precisely the same operative procedures as in the experiments with reserpine. The concentration of noradrenaline was 5·0 ± 1·5 on the left side, and 5·0 ± 0·6 μg/g on the right. In another control experiment, chlorpromazine hydrochloride (10 mg/kg intravenously) was injected into four rabbits; these were killed 3·5 hr later, when they showed heavy sedation and low body temperature. The noradrenaline concentration of the cervical ganglia removed after the drug had been allowed to act was normal (4·9 ± 1·6 μg/g). Another control with morphine injections will be found in the section on cats.

Animals injected with reserpine exhibit miosis, narrowing of the palpebral fissure and relaxation of the nictitating membrane. To decide whether these signs might be caused or contributed to by the loss of transmitter from the adrenergic neurones, the peripheral end of the severed cervical sympathetic chain was stimulated electrically before and after administering reserpine in a series of rabbits; pupil and lid movements were observed (the nictitating membrane of the rabbit is unsuitable for observation), and the noradrenaline content of the superior cervical ganglia was estimated. That it was permissible to use the same ganglion for stimulation and extraction follows from previous experiments in the dog (Vogt, 1954), in which it was shown that stimulation of the cervical sympathetic trunk, even for periods of 2 hr and more, did not change the concentration of noradrenaline in the ganglia.

In eighteen rabbits, in which the noradrenaline concentration was above 1 μg/g, responses to stimulation of the cervical sympathetic were not, or only slightly, impaired by reserpine. When, however, the concentration in the right cervical ganglion had fallen below 1 μg/g, the result varied with the duration of the experiment. Of eight rabbits tested about 17 hr after the injection, four showed no response of pupil or eyelid when the pre- or post-ganglionic fibres of their right ganglia were stimulated with currents which were between 3 and 13 times stronger than those with which good responses on the left side had been obtained before reserpine. In the four other rabbits, in which the strength of the stimuli was pushed further, very small and sluggish responses were obtained, but could not be made brisker or larger by increasing the strength of the current.

When, however, responses to stimulation of the cervical sympathetic were tested soon (between 2·5 and 4 hr) after the injection of reserpine, even severe depletion of the noradrenaline of the superior cervical ganglia did not impair the responses. Thus a fall in noradrenaline concentration to 0·3 μg/g was compatible with normal electrical thresholds (three rabbits).

That any loss of response of the smooth muscle to stimulation of the adrenergic nerves is due to the loss of transmitter can only be assumed if the excitability of the smooth muscle itself is not reduced by reserpine. To check this point, intravenous injections of noradrenaline were made into reserpinized rabbits, and the effect on the blood pressure was recorded. The pressor responses of such animals were either normal or exaggerated, never diminished. This is in good agreement with previous reports of enhanced responses to adrenaline and noradrenaline of the blood pressure and the nictitating membrane of the cat (Bein, Gross, Tripod & Meier, 1953).

TABLE 2. Effect of reserpine on the adrenaline concentration (μg/g fresh tissue) in the superior cervical ganglia of rabbits. (Left ganglion before, right after reserpine)

			Adrenaline		
No. of rabbits	Dose of reserpine (mg/kg)	Interval (hr)	Left (μg/g)	Right (μg/g)	Change (%)
1	2·3	15	0·50	0·50	0
3	2·1	16	0·13	0·20	+54
3	1·4	16	0·28	0·56	+96
1	0·8	18	0·40	0·85	+112
2	0·3	18	0·51	0·36	−29
			Mean 0·34	0·49	

The injections were made intraperitoneally. The interval refers to the time between injection and excision of the right ganglion.

In a single rabbit (no. 29, Table 1) iproniazid (100 mg/kg I.V.) was injected 1 hr before reserpine. Brodie & Shore (1957) have shown that, after the injection of this inhibitor of amine oxidase, reserpine produces a much smaller loss of 5-HT from the brain. The noradrenaline loss from the cervical ganglia, however, was severe, and the question of a possible protection of noradrenaline by iproniazid was not followed up.

Estimations of the adrenaline content of superior cervical ganglia of rabbits were difficult because of the minute quantities involved (between 1 and 7 ng/ganglion). Table 2 shows that, in five experiments on ten rabbits, there was no evidence of any fall in the adrenaline concentration after doses of reserpine which depleted the ganglia of their noradrenaline. We shall return to this point later.

Cats. Experiments on the superior cervical ganglia of cats confirmed the observations in rabbits. The results are summarized in Fig. 1. The noradrenaline concentration was 3·5 ± 0·3 before, and 0·6 ± 0·1 μg/g on the day following, an injection of reserpine (1–2·5 mg/kg). In contrast to the rabbit, it made no difference whether the drug was given intravenously or intraperitoneally. The effect appeared to be as great after 1·3 as after 2·5 mg/kg.

The response of eyelid, pupil and nictitating membrane to electrical stimulation of the pre- and post-ganglionic fibres was tested in five normal and in three reserpinized cats. In one of the treated cats there was no response, and the threshold was greatly raised in the other two. Over 80 % of the noradrenaline had disappeared from their ganglia. In the most severely affected animal the noradrenaline content was 0·36 μg/g.

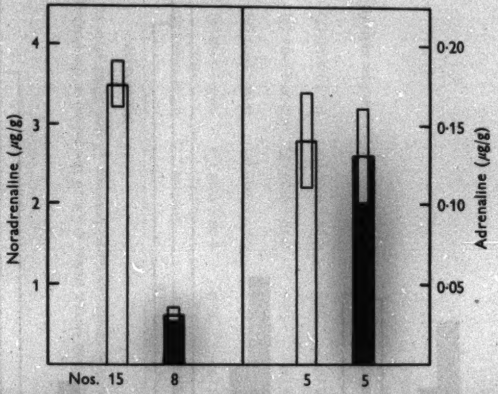

Fig. 1. Noradrenaline and adrenaline concentrations in the superior cervical ganglia of cats (mean and s.e. of the mean). Open columns, control figures. Black columns, results obtained between 13 and 20 hr after the intravenous injection of reserpine (1·0–2·5 mg/kg). Two cats were injected intraperitoneally. Number of cats at the foot of each column.

Many drugs are known to deplete the brain of its noradrenaline (Vogt, 1954); with the exception of reserpine they are all drugs which produce signs of great sympathetic activity. These drugs had not been tested on the peripheral sympathetic system before, and therefore the effect of morphine, a violent sympathetic stimulant in the cat, was compared with that of reserpine on the amine content of brain and of peripheral sympathetic tissues. Fig. 2 illustrates the results. Whereas the concentration of noradrenaline was low everywhere after reserpine, depletion after morphine only occurred in the hypothalamus and not in the cervical ganglia or splenic nerves.

In the cat, as in the rabbit, there was no evidence that reserpine produced changes in the adrenaline concentration of the superior cervical ganglion; the mean content after reserpine was only 7% less than that of the normal cat (Fig. 1).

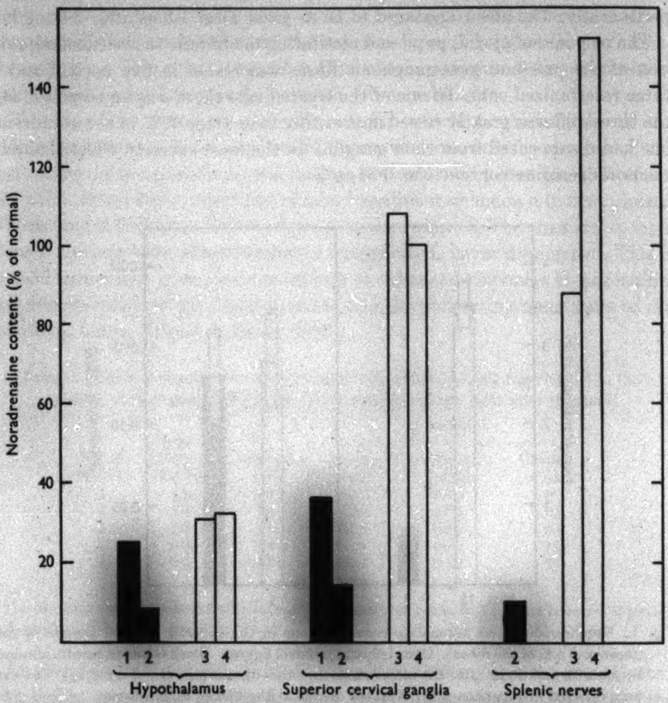

Fig. 2. Noradrenaline content of hypothalamus, superior cervical ganglia, and splenic nerves of each of four cats, as percentage of normal mean. Black columns (cats 1 and 2) after reserpine (1 and 2 mg/kg) intraperitoneally. Open columns (cats 3 and 4) after morphine HCl (40 and 35 mg/kg) subcutaneously. Number of cat at the foot of each column. Only the hypothalamus is affected by both drugs.

Stellate ganglia, thoracic and abdominal chains, splenic nerves

These parts were examined only in the cat. The stellate ganglia are anatomically strictly analogous to the superior cervical ganglia. Their normal concentration of noradrenaline was slightly, but not significantly, lower than that of the cervical ganglia, and so was that of adrenaline. As in the cervical ganglia, reserpine (1·0–2·0 mg/kg) caused an average fall of noradrenaline to 15%, whereas the concentration of adrenaline remained unchanged (Table 3).

TABLE 3. Concentration of noradrenaline and adrenaline (μg/g fresh tissue, mean ±S.E. of the mean) in the stellate ganglia, the thoracic and abdominal chains, and the splenic nerves of cats. Normal animals and animals examined between 13 and 20 hr after an intravenous injection of reserpine

		Noradrenaline			Adrenaline			Percentage methylated	
Tissue	Dose range of reserpine (mg/kg)	Control (μg/g)	After reserpine (μg/g)	Amine left (%)	Control (μg/g)	After reserpine (μg/g)	Amine left (%)	Before reserpine	After reserpine
Stellate ganglia	1·0–2·0	2·9±0·4 (9)*	0·43±0·08 (7)	15·0	0·09±0·03 (7)	0·09±0·03 (5)	100	2·4	21
Thoracic chains	1·5–2·5	0·71±0·06 (4)	0·11±0·03 (5)	15·5	0·02±0·01 (3)	0·04±0·01 (5)	200	2·7	27
Abdominal chains	2·5	1·6±0·1 (4)	0·20±0·04 (4)	12·5	0·10±0·03 (2)	<0·06±0·01 (4)	<60	5·9	<23
Splenic nerves	1·0–2·5†	2·5±0·4 (9)	0·64±0·16 (9)	26·6	0·23±0·06 (6)	0·13±0·04 (7)	57	8·5	17

The last columns give the percentage of adrenaline in the mixture of the two catecholamines.

* Number of cats in parentheses.

† One cat injected intraperitoneally.

Essentially similar results were obtained for the remaining parts of the paravertebral sympathetic system, i.e. the thoracic and lumbar chains. In these, however, the ganglia were not separated from the fibres with which they form the chain. Owing to the large admixture of medullated non-adrenergic components the amine concentrations in the chains were lower than in pure ganglionic tissue (Table 3). As before, injections of reserpine (1·5–2·5 mg/kg) greatly lowered the concentration of noradrenaline without producing significant changes in the content of adrenaline.

Table 3 also contains estimations carried out on the splenic nerves. These provided a specimen of post-ganglionic sympathetic fibres containing little, if any, ganglionic tissue. The normal concentration of noradrenaline, 2·5 ± 0·4 μg/g was barely significantly lower than that in cervical ganglia; reserpine lowered it to about a quarter of normal. The adrenaline figures suggested occasional lowering by reserpine; this would seem in contrast to the data obtained on paravertebral ganglia, and bring the splenic nerves in line with the solar ganglion, from which they originate (see below); the difference, however, in the mean adrenaline concentration before and after reserpine is not significant.

TABLE 4. The effect of decentralization of the left superior cervical ganglion on the action of reserpine

	Interval (days)	Dose of reserpine (mg/kg)	Noradrenaline* Left side (μg/g)	Noradrenaline* Right side (μg/g)
Cat 1	12	0	2·7	3·1
Cat 2	19	0	3·5	3·4
Cat 3	12	1·5	0·42	0·41
Cat 4	17	1·5	0·32	0·49
Rabbit 1	3	1·2	1·5	1·4
Rabbit 2	3	1·2	1·4	1·5

The interval refers to the time between decentralization of the left ganglion and the experiment. Reserpine was given intravenously between 13 and 17 hr previous to the dissection of the ganglia.

* μg/g fresh tissue.

In view of the fact that denervation renders the adrenal medulla of the cat, and to some extent that of the rabbit, refractory to reserpine (Holzbauer & Vogt, 1956; Kroneberg & Schümann, 1957), experiments were carried out to test the effect on the action of reserpine of decentralization of the superior cervical ganglion. In four cats and two rabbits the left superior ganglion was severed from its connexions with the central nervous system by removal of a piece of the preganglionic nerve trunk. It will be seen (Table 4) that reserpine caused the same depletion in the left (decentralized) and in the right (normal) ganglia of both cats and rabbits. The average loss of noradrenaline was 88% in the cats and 68% in the rabbits. In order to ascertain that decentralization as such does not change the noradrenaline content of ganglia, cats 1 and 2 were subjected to decentralization of the left superior cervical ganglion and killed, without being given reserpine, 12 and 19 days later. There was no significant difference between the noradrenaline content of the left and right ganglia. Thus, up to 19 days at least, the concentration of transmitter in the adrenergic neurone is not affected by a lack of impulses discharging along it, nor by the degeneration of the preganglionic fibres.

The observation that the effect of reserpine on the sympathetic ganglia persists after decentralization agrees well with the finding (Brodie, Olin, Kuntzman & Shore, 1957) that the sympathin of the heart is lost when reserpine is given to rabbits in which the cord has been divided.

Prevertebral ganglia

Rabbits. Estimations were carried out on the solar and the inferior mesenteric ganglia. The concentration of catecholamines in these ganglia is much higher than in the paravertebral sympathetic chains, and higher in the inferior mesenteric than in the solar ganglion. In contrast to what will later be shown in the cat, the range, and therefore the standard error, was large only in the inferior mesenteric ganglion. The normal concentrations and those determined after the injection of reserpine will be found in Table 5. The 'normal' rabbits actually consisted of five untreated animals and of four animals injected with chlorpromazine. It has been shown above that chlorpromazine does not alter the noradrenaline content of the superior cervical ganglia, and the same was found to hold for the prevertebral ganglia. For the purpose of Table 5 the rabbits injected with chlorpromazine were therefore classified as 'normal'. No figures for the adrenaline concentration in the inferior mesenteric ganglia are included, since the tissue available weighed only a few milligrams and estimations were rarely successful.

Row 1 contains the figures for normal rabbits; the high concentration of adrenaline in the solar ganglion is in striking contrast to the low values obtained in all paravertebral ganglia (Tables 2 and 3). When reserpine was given in a single dose of 0·1 or 0·2 mg/kg and the rabbit was killed on the following day (rows 2 and 3, Table 5), there were convincing losses of catecholamines only with the larger dose. In the following eight experiments, still larger doses (1·0–1·6 mg/kg) were injected (row 4, Table 5); the ganglia were excised not less than 14 hr after the injection. The noradrenaline concentration fell in all ganglia by approximately the same amount, and so, apparently, did the adrenaline concentration in the solar ganglion. This is in contrast to the refractoriness of the adrenaline contained in the paravertebral system. Examination of the figures, however, shows that the individual adrenaline concentrations have such a large scatter that the mean content after reserpine is not significantly different from that before reserpine. But since four of the eight values are well below the normal range, the right conclusion to draw appears to be that reserpine does lower the concentration of adrenaline, but

TABLE 5. Catecholamine concentrations (µg/g fresh tissue, mean ±S.E. of the mean) in the prevertebral ganglia of rabbits. Effect of intravenous injections of reserpine

Group no.	Dose of reserpine (mg/kg)	Interval (hr)	Solar ganglion: Noradrenaline (µg/g)	Solar ganglion: Noradrenaline Lost (%)	Solar ganglion: Adrenaline (µg/g)	Solar ganglion: Adrenaline Lost (%)	Inferior mesenteric ganglion: Noradrenaline (µg/g)	Inferior mesenteric ganglion: Noradrenaline Lost (%)	Superior cervical ganglion: Noradrenaline lost (%)
1	Controls	0	6·7±0·7 (7)*	0	1·5±0·4 (9)	0	8·6±1·5 (8)	0	0
2	0·1	16	5·2 (1)	23	0·6 (1)	61	3·6 (1)	58	2
3	0·2	17	1·9 (1)	72	<0·5 (1)	67	3·3 (1)	62	59
4	1·0–1·6	14–17½	1·8±0·3 (8)	73	0·6±0·2 (8)	60	2·8±0·7 (6)	67	80
5	14 or 18 × 0·1, 8 × 0·2	—	1·3±0·1 (6)	80	0·13±0·02 (6)	91	1·4±0·3 (4)	84	80
6	1·6	¼–2½	2·4±0·4 (8)	64	1·0±0·3 (6)	33	8·2±2·1 (6)	4·7	70
7	1·6	4	0·49±0·06 (3)	93	0·29±0·08 (3)	81	6·9±3·7 (3)	20	94

* Figures in parentheses number of experiments; most figures on inferior mesenteric ganglia, and some on other ganglia, were obtained on the pooled tissue of two rabbits, and counted as one experiment.

that some ganglia have such a high initial adrenaline content that even after depletion the content may greatly exceed low normal values.

Row 5 shows the effect of repeated small doses of reserpine. A total amount of approximately 1·6 mg/kg, which depletes the ganglia when given in one injection, was given in divided doses spread over 8–18 days. The noradrenaline concentration of all ganglia was reduced to about 20% and so was the adrenaline content of the solar ganglion. Thus this treatment was at least as effective as the single dose in depleting the ganglia of their noradrenaline, and, in addition, produced a highly significant effect on their content of adrenaline.

In many rabbits electrical stimulation of the hypogastric nerves was carried out after administering reserpine. Even in animals in which the cervical sympathetic had lost so much transmitter that the response of the eyelid and pupil was absent, the vas deferens contracted on stimulation of the hypogastric nerves with minimal currents. The concentration of noradrenaline in the inferior mesenteric ganglion, from which the hypogastric fibres originate, was below 1μg/g in only one of these experiments, and perhaps there was always enough transmitter left to stimulate the effector organs. Another possibility is the presence of cholinergic fibres coursing to the vasa deferentia in the hypogastric nerves. This possibility was rendered very unlikely by the results of an experiment in which the excitability of the centrally cut hypogastric nerves of a normal anaesthetized rabbit was tested before and after the administration of eserine and of atropine. First 0·2, and later 0·4 mg/kg eserine sulphate was injected intravenously. The eserine caused defaecation and violent spasms of the colon, but responses of the bladder and vas deferens to stimulation of the hypogastric nerves were obtained at the same voltage and were not increased in size. Atropine sulphate (5·5 mg/kg intravenously) was then given. This completely relaxed the colon but was without effect on the threshold of the responses of bladder neck and vas deferens to indirect electrical stimulation.

Cats. In normal cats the catecholamine concentration in the prevertebral ganglia is much more variable than in the rabbit. This is particularly true of the inferior mesenteric ganglion, in which the figures for noradrenaline ranged from 9·6 to 46·2μg/g and those for adrenaline from 0·4 to 15·6μg/g. Large concentrations of adrenaline were found only when that of noradrenaline too was high. There is little doubt that these high and variable concentrations result from the occurrence in these ganglia of chromaffine tissue. Its development and distribution has been described by Kohn (1903). Kohn states that all sympathetic ganglia contain some chromaffine tissue, but it is obvious from his drawings that the largest chromaffine bodies invade the prevertebral ganglia on the ventral aspect of the aorta. In a few instances we have looked for chromaffine tissue in the stellate, the inferior mesenteric and the solar ganglia of cats. The tissue was fixed in a mixture of bichromate and formaldehyde, cut and stained with haematoxylin. Chromaffine bodies were found in the inferior mesenteric and solar ganglia, but not in the stellate ganglia. This would agree with the findings of low concentrations of adrenaline in the stellate ganglion and high values in the prevertebral ganglia. Since the chromaffine tissue usually lies on the surface of the ganglia and its size and shape is very variable, the amount included in the excised ganglia is bound to vary, and this may explain the large range in amine content.

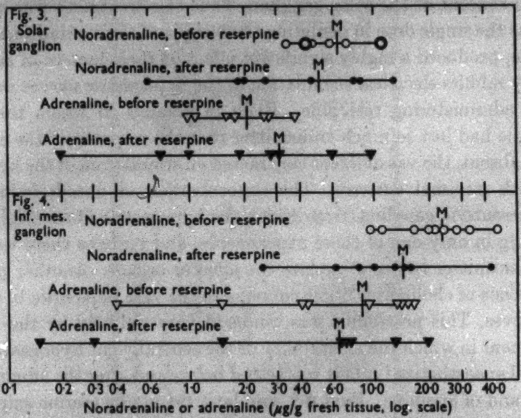

Fig. 3. Catecholamines in the solar ganglia of individual cats. Normal concentrations (open signs); and content after reserpine, 1·0–2·5 mg/kg, usually intravenously (black signs). Excision of ganglia 13–17 hr after the injection. M, mean.

Fig. 4. Catecholamines in the inferior mesenteric ganglia of individual cats. Normal concentrations (open signs); and content after reserpine, 1·0–2·5 mg/kg, normally intravenously (black signs). Excision of ganglia 13–20 hr after the injection. M, mean.

Figs. 3 and 4 illustrate the findings on normal cats and on cats given reserpine intravenously on the day preceding the extirpation of the ganglia. With doses higher than 1·0 mg/kg the results bore no relation to the size of the dose. It will be seen from the figures that the mean concentrations of catecholamines after reserpine did not differ significantly from the control means. Yet Figs. 3 and 4 show that an effect was, indeed, produced. It is manifested by the fact that, after reserpine, amine concentrations occur which are considerably below the lowest normal figures. Thus the lowest concentration of noradrenaline in the normal solar ganglia was 3·4 μg/g, but four of eight reserpine-treated cats had lower concentrations (0·6–2·6 μg/g). The effect on the mean was masked by the excessively high concentration of 12·7 μg/g found in one of the reserpinized cats.

The question may be asked why this difficulty was not encountered in the rabbit, the solar ganglion of which showed a consistent reduction in the concentration of both catecholamines. The difference can be explained if one assumes that in both species chromaffine tissue in the ganglia behaves like adrenal medullary tissue. First, there is practically no noradrenaline in the adrenal medulla of rabbits, whereas large and variable amounts are found in the cat. In the rabbit the presence of chromaffine tissue should therefore hardly contribute to the noradrenaline content of the solar ganglion, and reserpine should deplete this tissue of its noradrenaline in the same consistent way as it depletes paravertebral ganglia. Secondly, the amines in the adrenal medulla of rabbits are much more susceptible to the action of reserpine than the amines of cat adrenals (see next section). This would explain why, in the rabbit, the chromaffine tissue loses its adrenaline as easily as the adrenergic neurones their noradrenaline. Thus, after larger and particularly after repeated doses of reserpine, noradrenaline and adrenaline disappear to about the same extent from the solar ganglia (Table 5). Only in experiments of short duration was there greater loss of noradrenaline than of adrenaline from the solar ganglia. In the cat, on the other hand, the large depletion of noradrenaline from the neurones may be masked by the localization of some of the noradrenaline in chromaffine tissue, from which it is not very readily lost.

Stimulation of the hypogastric nerves was also attempted in cats, but, in contrast to the rabbit, contractions of the vas deferens were difficult to obtain in normal animals, and contraction of the bladder was seen at a wide range of thresholds in different individuals. It was therefore not possible to decide whether the treatment with reserpine had altered the response of the organs to electrical stimulation of the nerves.

Dogs. A few experiments were carried out in the dog, in which the hypogastric nerves are thick enough for analysis and in which the inferior mesenteric ganglia are very large. The hypogastric nerves, which normally contained $2{\cdot}2 \pm 0{\cdot}4\,\mu g/g$ of noradrenaline (range of five determinations 1·7–3·6), and a very variable amount of adrenaline (range 0·09–1·23 $\mu g/g$), had lost 80% of their noradrenaline 15 hr after a single dose of reserpine (0·5 mg/kg), and 91% after four doses (one injection per day intravenously, 0·5 mg/kg on the first and last, 0·25 mg/kg on the remaining days). The adrenaline content remained within the (very wide) normal range. Electrical stimulation of the centrally cut hypogastric nerves normally caused peristaltic movements and blanching of the vasa deferentia as well as contraction of the bladder neck, but after the single dose only bladder movements occurred, and after the four doses all responses had vanished. The noradrenaline concentration had fallen to 0·48 $\mu g/g$ in the first and to 0·2 $\mu g/g$ in the second instance.

In the mesenteric ganglia, normally found to contain amounts of noradrenaline and adrenaline which varied but little and greatly exceeded those of cats and rabbits, reserpine caused losses which were large for noradrenaline and only slightly less severe for adrenaline. Thus the normal concentrations were 40·2 ± 7·0 μg/g noradrenaline and 39·0 ± 5·3 μg/g adrenaline; after the single dose of reserpine the noradrenaline had fallen by 88 %, the adrenaline by 83 %; the repeated doses caused a loss of 91 % of the noradrenaline and of 80 % of the adrenaline, with corresponding tissue concentrations of 3·6 μg/g noradrenaline and 7·7 μg/g adrenaline. These values are higher than even the normal concentrations in the hypogastric nerves, and there is little doubt that they represent what was left of the catecholamines of the chromaffine tissue. The loss of response to stimulation and the low noradrenaline content (0·2 μg/g) of the hypogastric nerves indicate that this fraction of the catecholamines is not available as transmitter.

Adrenal medulla

Rabbits. Carlsson & Hillarp (1956) observed that a large dose of reserpine (5 mg/kg) almost completely emptied the rabbit's adrenal of its catecholamines. Kroneberg & Schümann (1957) found that 90 % of the adrenaline disappeared after 2 mg/kg. They also state that denervation reduces but does not prevent the loss. We had the same experience in one rabbit, in which 24 hr after reserpine (1·6 mg/kg) the adrenaline concentration had fallen to 95 % of normal on the innervated side, and to 75 % on the denervated side. In this experiment, however, the more severely affected innervated adrenal contained actually twice as much noradrenaline (1·4 μg/g) as the denervated adrenal. The percentage of methylated amine was thus a little lower than normal (95 instead of 98 %), a shift which had not been seen in experiments lasting only 4 hr. This observation was followed up in a series of experiments, in which the same total quantity of reserpine was given in divided doses over a period of many days. Table 6 contains the results. All adrenals had lost over 99 % of their adrenaline, but relatively less noradrenaline, and the percentage of the methylated amine ranged from 43 to 76 % instead of the normal 98 %. Since the amount of noradrenaline found was always much lower than the normal concentration, these experiments might be interpreted on the assumption that adrenaline is lost more readily than noradrenaline. It is, however, more likely that the relative excess of noradrenaline indicates that resynthesis is proceeding even while the reserpine is acting, and that the process of methylation is slower than the manufacture of the noradrenaline molecule.

It was stated earlier that an amount of reserpine given in divided doses over a period of 2 weeks is as effective in depleting noradrenaline, and more effective in depleting adrenaline, from ganglia than the same quantity injected as a single dose (Table 5). Table 6 shows that the loss of medullary amines after repeated small doses of reserpine is much more complete than after a single dose, when the loss did not exceed 95 %.

Cats. Previous experience had suggested that the adrenal medulla of the cat is more resistant to reserpine than that of the rabbit. With doses of

0·4 mg/kg no depletion of total amines had been found in the denervated, and an average loss of only 41% in the innervated, gland (Holzbauer & Vogt, 1956). In order to check whether this was only a question of dosage, seven cats with one denervated adrenal were given larger amounts of reserpine (1·0–2·5 mg/kg). In five of these cats (1–5, Table 7) the concentration of total amines on the denervated side lay within the range of 0·48–1·56 mg/g found in cats which were not injected with reserpine. It would, therefore, appear that doses up to 2·5 mg/kg do not affect the denervated adrenal. The loss on the innervated side was 54%, a little greater than with the dose of 0·4 mg/kg, and much smaller than the corresponding figure in rabbits, which is at least 90%. In two cats, however (nos. 6 and 7), obvious depletion was obtained in both adrenals, the figure for the denervated adrenals falling well below the lowest control figure. There was also very little difference in the concentration

TABLE 6. Effect of repeated intravenous injections of reserpine on the medullary amines (μg/g fresh adrenal) of the rabbit

					Difference from normal	
Individual dose (mg/kg)	No. of doses*	Nor-adrenaline (μg/g)	Adrenaline (μg/g)	Methylated (%)	Nor-adrenaline (%)	Adrenaline (%)
5 Controls	0	9·4† ±1·2	501† ±58	98	0	0
0·2	8	0·7	0·5	43	93	99·9
0·2	8	1·6	2·0	56	83	99·6
0·1	14	2·3	7·3	76	76	98·5
0·1	14	0·7	1·4	67	93	99·7
0·1	18	1·2	2·5	68	87	99·5
0·1	18	0·4	0·9	68	96	99·8

* One injection per day.

† Mean ±S.E. of the mean.

TABLE 7. Effect of a single, large intravenous dose of reserpine on the concentration of adrenaline and noradrenaline in the left (denervated) and the right (innervated) adrenal medulla of the cat

			Left (denervated) adrenal		Right (innervated) adrenal		
Cat no.	Reserpine (mg/kg)	Interval between denervation and expt. (days)	Catecholamines (mg/g)	Methylated (%)	Catecholamines (mg/g)	Methylated (%)	Catecholamines lost from innervated gland* (%)
1	1·0†	23	1·16	85	0·57	65	69
2	1·5	12	1·20	64	0·65	64	53
3	2·5	16	0·52	73	0·30	70	39
4	2·5	16	0·63	75	0·30	60	47
5	2·5	24	0·96	67	0·24	54	71
6	2·0	29	0·23	72	0·17	71	
7	2·5	26	0·26	57	0·18	48	

The catecholamines of the denervated adrenal of 18 cats not injected with reserpine ranged from 0·48–1·56 mg/g fresh tissue (mean 0·91 mg/g). The adrenals were removed between 15 and 19 hr after the injection of reserpine.

* Denervated gland = 100.

† Intraperitoneally.

of amine on the two sides. It seems likely that denervation was not satisfactory in these two cats. In one of them (no. 7), the lesser splanchnic nerve was not found at operation, so that incompleteness of denervation is likely to explain at least this result. Perhaps some re-innervation had taken place in the other. The ratio adrenaline to noradrenaline was not much affected by the reserpine; it fell a little in three and remained the same in two cats.

Time course of the action of reserpine

A single dose of reserpine (1·6 mg/kg) was given to rabbits, and sympathetic ganglia were examined at different intervals after the injection. The injection was intravenous when the experiments were of short duration, and intraperitoneal for the long-term experiments.

Onset of action

Figs. 5 and 6 illustrate the early phase of the depletion of noradrenaline from the different ganglia. The corresponding values for the adrenaline content of the solar ganglion are shown in rows 6 and 7, Table 5. It takes about 4 hr for the effect on the superior cervical and solar ganglia to reach its maximum, but more time is apparently required to deplete the inferior mesenteric ganglion. Fig. 6 represents all the noradrenaline estimations made of this ganglion, and shows the normal range and the individual estimations after reserpine; it illustrates the very large scatter both in the normal and in the treated rabbits. It is possible that what appears to be a greater resistance of the inferior mesenteric ganglion to reserpine is only an artifact resulting from the wide range of individual values.

Recovery

After a single injection of reserpine (1·6 mg/kg), the catecholamine content of the sympathetic system remains low for a long period of time. After 5 days (observations on four rabbits), the noradrenaline concentration of the superior cervical ganglia had recovered to just above half the normal value. Even after 8–9 days (three rabbits), the adrenals and the cervical, solar and inferior mesenteric ganglia contained reduced amounts of catecholamines, all figures but one lying below the normal range. Recovery was complete after 14 days (two rabbits) except for the adrenal medulla of one rabbit which was still partially depleted of its hormones; the ratio of adrenaline to noradrenaline was normal in the adrenals of both animals, as indeed it was in the incompletely restored glands examined after 8 and 9 days.

Other catecholamines

Dopamine (hydroxytyramine) has been demonstrated in sympathetic ganglia and splenic nerves of cattle by Schümann (1956). The quantity was of

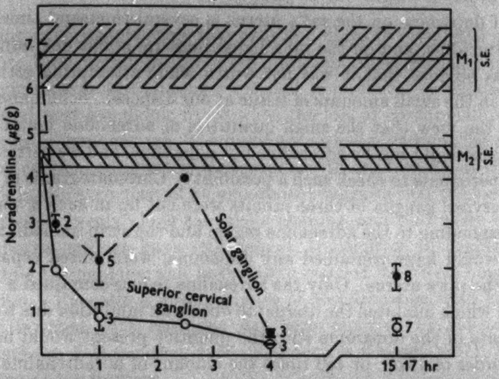

Fig. 5. Onset of effect of reserpine (1·0–1·6 mg/kg intravenously) on noradrenaline concentration in the solar and the superior cervical ganglia of the rabbit. The upper horizontal line (M_1) and hatched band represent mean and s.e. of the mean of the noradrenaline content of the solar ganglia of 7 normal rabbits, the lower horizontal line (M_2) and hatched area the corresponding figures for the superior cervical ganglia of 41 rabbits. ●---●, solar ganglia; ○—○, superior cervical ganglia of reserpinized rabbits. Vertical lines through points, s.e. of the mean. Number of experiments to the right of each point; when no s.e. is given, only one experiment was carried out. In most experiments, the superior cervical ganglia of two rabbits were pooled. Note that the effect of reserpine is fully developed at 4 hr.

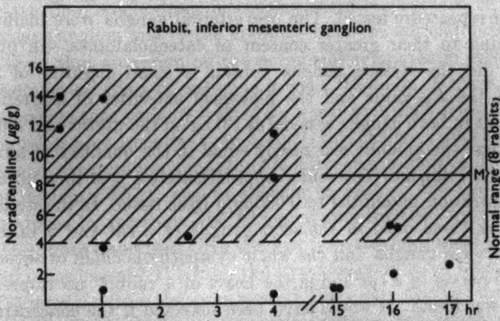

Fig. 6. Onset of effect of reserpine (1·0–1·6 mg/kg intravenously) on noradrenaline concentration in the inferior mesenteric ganglion of the rabbit. Hatched part of figure, normal range of noradrenaline concentrations (8 experiments). Continuous line M, mean normal value. Each dot represents one estimation on the ganglia of one or two reserpinized rabbits. Note that a convincing fall in noradrenaline was only obtained after 15–17 hr.

the same order of magnitude as that of noradrenaline. Since the biological activity of dopamine on the rat's uterus is several thousand times less than that of adrenaline, and on the rat's blood pressure about one hundred times less, it was obvious that it was not worth while attempting estimations of dopamine in the small amounts of tissue at our disposal. Schümann, however, expressed the view that the small quantities of adrenaline found in sympathetic ganglia might not be adrenaline but dopamine, and we carried out some control experiments to check such a possibility. Chromatograms of the pooled superior cervical ganglia of three rabbits were cut up in such a way that the strip corresponding to the adrenaline region and the strip immediately behind it, which would have contained any dopamine, were eluted separately and tested on the rat's uterus. Only the adrenaline region contained a heat-labile compound which inhibited the uterus; to obtain an inhibition due to dopamine by the eluate of the dopamine strip the quantity present would have had to be of the order of 10 μg, or 100 times the amount of noradrenaline present in the ganglia. Since Schümann found the quantities of noradrenaline and dopamine in cattle ganglia to be about equal, it is obvious that if the same held for the rabbit, amounts which could have been present in the eluates were far below the threshold of the assay.

Some experiments were done in which the isoprenaline region of chromatograms of sympathetic tissue from the cat was eluted and tested by parallel assay on the rat's uterus and the rat's auricles. In view of the work of Lockett (1957), who reported isoprenaline in the blood of cat heart–lung preparations when the thoracic chains were stimulated, the stellate ganglia and thoracic chains were repeatedly tested. The prevertebral ganglia were included in the survey owing to their greater content of catecholamines. In only one of four cats did the stellate ganglia contain a small quantity of a material which had the R_F value and the biological activities of isoprenaline. The total amount in both ganglia assayed as 1·0 ng on the uterus and 0·6 ng on the rat's auricles; the amount was nearly equal to the quantity of adrenaline in the ganglia (1·1 ng), and represented approximately 1 % of their noradrenaline. The concentration amounted to 0·035 μg/g fresh tissue. In the stellate ganglia of the other three cats, in the solar ganglia of a cat and a rabbit, in the superior cervical ganglia and the whole sympathetic chain of several cats, in the splenic nerves of a cat and in the heart of a rabbit, no isoprenaline-like substance was found. It would have been detected if the concentrations had reached levels which ranged from 0·003 to 0·01 μg/g of tissue. Under these circumstances the one positive finding of a concentration of 0·035 μg/g is insufficient evidence that isoprenaline is in fact stored in sympathetic tissue of the cat.

DISCUSSION

The foregoing experiments show that cells and fibres of peripheral adrenergic neurones lose their noradrenaline after injections of reserpine. Organs with an adrenergic innervation no longer respond to electrical stimulation of preganglionic and post-ganglionic fibres when the loss of noradrenaline is severe and has persisted long enough. The finding that low noradrenaline content of the superior cervical ganglion is compatible with excitability of the sympathetic trunk in the early part of an experiment suggests that the loss of amine does not proceed evenly along the whole neurone, and that there is a period when the nerve endings have still enough transmitter to enable them to function while the ganglion cell has already reached its lowest noradrenaline concentration. Recently a disappearance of noradrenaline from heart tissue of animals injected with reserpine has been reported by several authors (Bertler, Carlsson & Rosengren, 1956; Paasonen & Krayer, 1957); this agrees well with the above findings.

These observations pose the question whether the impaired function of the peripheral sympathetic system is a factor which contributes to the clinical picture produced by reserpine in man. The answer is suggested by the experiments in which small doses were injected over a long period of time, since it is under these conditions that the animal experiments are strictly comparable with the therapeutic procedures. Daily doses of the order 0·1 mg/kg are used in psychiatry, and these doses were shown to be as effective as single large doses in depleting sympathetic ganglia of rabbits of their noradrenaline, and more effective than single doses in causing the disappearance of adrenaline from the solar ganglion and of the hormone from the adrenal medulla. It is therefore likely that an action on the peripheral sympathetic system contributes to the over-all effect of reserpine in man. Gaddum, Krivoy & Laverty (1958) have shown that patients treated for weeks with daily doses of reserpine excrete much less noradrenaline than normal; this change might be caused by the reduction of the noradrenaline stores in adrenergic neurones. The difficulty remains of deciding which role to allocate to each of the many chemical changes produced by reserpine. Is the loss of noradrenaline from the sympathetic centres more important than the loss from the peripheral sympathetic system, and is the loss of 5-HT from the hypothalamus more important than the loss of sympathin from the same region? There may also be other chemical 'lesions' produced of which we are quite ignorant but which may affect function decisively.

Except for the special case of the prevertebral ganglia of the cat, the noradrenaline was readily lost from the sympathetic neurones of all species; this loss was independent of any connexions with the central nervous system. In contrast, there was no evidence that adrenaline was lost from any tissue in which its initial concentration was very low, such as paravertebral ganglia and post-ganglionic fibres. In the prevertebral ganglia, which contain high concentrations of adrenaline in what is probably chromaffine tissue, the loss was easily demonstrable in the dog and the rabbit, but not in the cat. The difference between the rabbit and the cat is even more pronounced with regard to the adrenal medullary amines. The adrenal medulla of the rabbit is very easily depleted by moderate doses of reserpine and denervation affords only a little protection. In the cat losses of medullary hormones from the innervated gland are very variable, and denervation affords a high degree of protection. The reason why connexion with and presumably stimulation by the central nervous system should be essential for the effect of reserpine to take place on some tissues and not on others is quite obscure, and perhaps points to different modes of binding of the amines at various sites.

Recovery of the normal concentration of amines after a single injection of reserpine took a long time, and usually more than 9 days. This is somewhat longer than the time required for the restoration of the 5-HT lost from brain tissue (Shore, Pletscher, Tomich, Carlsson, Kuntzman & Brodie, 1957). According to the same authors reserpine is not stored in tissue for more than a few hours. It is not clear how much of the time needed for restoration of the normal content of catecholamines is taken up by the period during which binding of the amines is impaired and how much simply represents the time needed for resynthesis. That the latter takes days is known from experiments with radioactive tracers (Udenfriend & Wyngaarden, 1956), and from the work on cat adrenals depleted of medullary hormone by acetylcholine (Butterworth & Mann, 1956). One interesting difference between the recovery process in the adrenal of the rabbit and that of the cat is the fact that the percentage of methylated amine is normal in the rabbit long before the total amine content is restored, whereas Butterworth & Mann found the opposite to be true of the cat, in which total amine concentration was back to normal while the percentage of adrenaline was still very low. This is a good example of the greater efficiency of the methylation process in the rabbit as compared with the cat.

Nevertheless, it was possible by repeated daily injection of reserpine to produce a rabbit adrenal containing a mixture of catecholamines in which the percentage of methylated amine was low. This finding suggests, but does not prove, that resynthesis of amines proceeds unimpeded during the presence of reserpine in the tissues, and that even in the rabbit methylation is a slow step in adrenaline synthesis.

During the early period of the action of reserpine some manifestation of the release of medullary amines and adrenergic transmitter into the circulation might be expected; and this is, indeed, seen when conditions are favourable. Thus Everett *et al.* (1957) described evanescent pilo-erection in mice or rats about half an hour after large doses of reserpine (50–100 mg/kg by mouth);

Kuschke & Frantz (1955) saw hyperglycaemia in the rabbit; tachycardia has been seen to occur in the heart-lung preparation of the dog (Plummer, Earl, Schneider, Trapold & Barrett, 1954), and many workers have observed rises in blood pressure in different species (de Jongh & van Proosdij-Hartzema, 1955; Maxwell, Ross, Plummer & Sigg, 1957; Domino & Rech, 1957). A raised level of blood adrenaline has been found in rabbits during the first hour after an intravenous dose of reserpine (Muscholl & Vogt, 1957*c*).

SUMMARY

1. Fibres and ganglia of adrenergic neurones of rabbits, cats and dogs lose a large fraction of their noradrenaline when the animals are injected with reserpine.

2. When the loss of noradrenaline is severe, and has persisted for more than four hours, it may lead to loss of response from the organs innervated by adrenergic fibres to indirect electrical stimulation.

3. The normal adrenaline content of the paravertebral sympathetic tissue is low, and shows no consistent changes after reserpine. In contrast, very high adrenaline concentrations are found in the prevertebral (solar and inferior mesenteric) ganglia of all species examined, evidently because of the presence in these ganglia of much chromaffine tissue. In the rabbit and in the dog treated with reserpine the adrenaline and the noradrenaline disappear as readily from the prevertebral ganglia as the noradrenaline disappears from the paravertebral ganglia. In the cat's prevertebral ganglia reserpine produces erratic changes in the concentration of both amines; whereas evidence of depletion is found in some, it is not seen in other individuals. This peculiar response of cat ganglia is explained on the assumption that the nervous tissue proper always loses its noradrenaline, but that the chromaffine tissue of cat ganglia is less readily depleted of its amines than the chromaffine tissue in the ganglia of rabbits, just as the adrenal medulla of the cat is more resistant to the action of reserpine than that of the rabbit. If we further assume that the percentage of methylated amine is as low in the ganglionic as it is in the medullary chromaffine tissue of cats, the resistance of the ganglia to the action of reserpine will affect all the adrenaline and that fraction of noradrenaline which is localized in the chromaffine cells.

4. The great efficacy of small daily doses of reserpine in causing a loss of catecholamines throughout the body suggests that chronic therapeutic administration of reserpine in man will also lead to a reduction in his stores of catecholamines.

Our thanks are due to Professor D. Whitteridge, F.R.S., who kindly checked the performance of our Ritchie Sneath stimulator; to Mrs Frances Bird and Mr David Flockhart for their help in our chemical analyses; to the Medical Research Council for a grant (to M. V.) towards the expenses of this work; to Ciba Laboratories, Ltd., for a generous supply of Serpasil and to Roche Products, Ltd., for a gift of Marsilid (iproniazid). The experiments were carried out during the tenure by one of us (E.M.) of a British Council Scholarship.

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THE HEAT EXCHANGES OF WET SKIN

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Machle & Hatch (1947) have examined theoretically the effect of skin temperature and skin wetness (Gagge, 1937) on the heat exchange in warm environments. Their equation giving the steady state skin temperature in terms of the metabolic rate and the relevant parameters of the environment was designed to cover the full range of warm environments, but proved unusable in most circumstances because the skin wetness could not be determined. Skin wetness can be calculated from skin temperature on the basis of the equations, but can be independently stated only in the case of complete wetness, that is, when the skin is covered with a film of sweat. In this condition the term for skin wetness becomes unity, and the steady state skin temperature can be predicted from a knowledge of the metabolic rate and the environment (Woodcock, Pratt & Breckenridge, 1952).

The work to be described was an attempt to provide a direct check of this theoretical approach. In practice it was found that the steady state could not be reached in the severe environments required to ensure full wetness of the skin, and recourse was had to the prediction previously made by Machle & Hatch of the rate at which the skin temperature should approach its equilibrium value. Their formulae include certain secondary statements or assumptions which are incidental to the main thesis. These have been examined directly, and the equations have been modified to avoid the inclusion of those statements which were found inapplicable to the situation used in the present work.

Derivation of the formulae for the prediction of the temperature of the wet skin

The approach used by Machle & Hatch to the expression of the temperature of wet skin either in the steady state or before this has been reached is here restated, so that the context of the alterations which have been found necessary may be made clear.

The rate of heat gain by unit area of skin from the environment by convection and radiation may be expressed by

$$H_{cr} = k_{cr}\,(T_a - T_s),$$

where the constant k_{cr} is the sum of the constants k_c, for heat exchange by convection, and k_r, for heat exchange by radiation, T_a is the temperature of the air and walls, and T_s that of the skin.

Where the skin and wall temperatures do not vary greatly and wall temperature equals air temperature, there is little error in assuming this linear relationship for heat exchange by radiation and convection.

The rate of heat loss by evaporation from unit area of wet skin is given by

$$H_e = k_e\,(E_s - E_a),$$

where k_e is the constant for heat loss by evaporation (expressed in heat units) and E_s and E_a are the vapour pressures at the skin surface and in the ambient air.

The vapour pressure at the skin surface is taken as 0·2 mm Hg less than that of pure water at skin temperature, to allow for the dissolved substances in the sweat. It is supposed that the sweat rate considerably exceeds the rate of evaporation so that there is no significant concentration as a result of the evaporation. Over the small skin temperature range covered by the experiments to be described, the water vapour pressure at the skin surface may be expressed as a linear function of temperature;

$$E_s = 2{\cdot}6 \times T_s - 49{\cdot}3,$$

(error 0·06 mm Hg at 35° C and 0·14 mm Hg at 39° C). The above expression differs from that used by Machle & Hatch, who were considering a range of skin temperature greater than that which was covered by the present experiments and having a lower mean value.

These formulae may be combined to give an expression of the skin temperature at which the metabolic heat production, M (per unit area) would be exactly dissipated.

$$T_s^* = \frac{k_{cr}T_a + k_e\,(E_a + 49{\cdot}3) + M}{(2{\cdot}6 \times k_e + k_{cr})}. \tag{1}$$

The expression has the general form of equation (4) of Machle & Hatch (1947), but differs from it in the following ways. The numerical constants are slightly different because of the different range of skin temperatures considered (see above). All area terms have been omitted by expressing the metabolic heat production per unit area and assuming that the skin is wet, so that the areas for heat exchange by convection and evaporation are equal. The term for wall temperature has been omitted, since in the present work air and wall temperatures were equal. It might appear that there is here an implicit assumption that the areas available for heat loss by radiation and convection are equal. Allowance is in fact made for this in the value assigned to k_{cr}. In the present work this was experimentally determined, and the contribution of the true radiation constant to the whole coefficient is automatically weighted by the ratios of these two areas. Heat exchange in the respiratory tract has been neglected, since this must have been small in the environments used here (McCutchan & Taylor, 1951).

Machle & Hatch (1947) have expressed the relation between skin temperature and time for constant skin wetness in the following way:

$$(T_s^* - T_s) = (T_s^* - T_0)\exp\left(-\frac{AC'}{SW\,(an+b)}\,t\right).$$

Here T_s^* = final skin temperature in the steady state; T_s = skin temperature at time t; T_0 = skin temperature at time 0; A = body surface area; $C' = 2{\cdot}5\ k_e + k_{cr}$; S = specific heat of body; W = weight of body; t = time.

$(an+b)$ represents the ratio of the change in mean body temperature to the change in skin temperature. Change in mean body temperature is defined as the weighted mean of skin and deep temperature changes $(a\Delta T_r + b\Delta T_s)$. It is supposed that the two temperatures will be linearly related over the relevant range so that $\Delta T_r = n\Delta T_s$. Since a and b are weighting factors, their sum is unity. It was found in the present experiments that, over the range examined, n was near to unity (see results), so that $(an+b)$ also has this value, and the difficulty of allocating values to a and b does not have to be faced. The value of 2·5 in the expression for C' given by Machle & Hatch is derived from the linear relation used by them for saturated water-vapour pressure in terms of temperature. For the present case 2·6 represents a closer approximation.

For the circumstances used here, therefore, the following expression should represent the relation between skin temperature and time when the skin is completely wet.

$$(T_s^* - T_s) = (T_s^* - T_0)\exp\left(-\frac{A(2\cdot6 \times k_e + k_{cr})}{SW}\,t\right). \tag{2}$$

The linear approximation for the relation between temperature and water-vapour pressure is accurate to within 0·1 mm Hg over the range of observed skin temperatures. When the final skin temperatures are above 39° C the assumed relation introduces some error into their calculation, but this does not affect the calculation of the rates of change of skin temperature within the range of the observations.

METHODS

All experiments were conducted in the same position in an air-conditioned room. During any one experiment air temperature was constant to ±0·25° C and relative humidity to ±0·5% r.h. The air circulated from ceiling distributors to extraction orifices at the floor level running along opposite sides of the room. Air movement in the region occupied by the subject was between 45 and 55 ft./min (katathermometer), and great care was taken to avoid varying this by rapid movement of the observer or subject. The aspirating psychrometer used for measuring the environmental temperature was run continuously for the same reason.

The subject reclined on a deck chair with a leg rest, the canvas being replaced by a wide-mesh nylon net. His posture was one of semirecumbency with the buttocks low and the lower legs horizontal and at about the level of the xiphisternum. The chair was arranged on the platform of a recording balance, since this was required for the determination of the heat exchange constants.

Skin temperatures were measured by an end-to-end thermocouple, lightly tensed, which was applied to each point in turn with sufficient pressure to produce slight deformation of the skin. This was calibrated during each experiment. Mouth temperature was measured with the same couple, detached from its mount and placed under the tongue. Mouth breathing was prohibited. Skin temperature was measured at the following points:

1, dorsum of foot;
2, anteromedial aspect of leg midway between knee and ankle;
3, posterolateral aspect of leg midway between knee and ankle;
4, medial aspect of thigh midway between perineum and knee;
5, anterolateral aspect of thigh midway between perineum and knee;
6, posterolateral aspect of thigh midway between perineum and knee;
7, abdomen over rectus at the level of the umbilicus;
8, abdomen in mid-axillary line between costal margin and ilium;
9, back, at margin of erectores spinae at the same level as 8;
10, chest 1·5 in. medial to nipple;
11, back over supraspinatus;
12, anterolateral aspect of upper arm midway between shoulder and elbow;
13, posterior aspect of forearm midway between elbow and wrist;
14, dorsum of hand;
15, forehead.

These points were chosen so that different regions of the body were represented by numbers of points approximately proportional to their areas, and an unweighted mean of the readings could be used for the expression of mean skin temperature.

When metabolic rate was measured, mouth temperatures were not taken. The subject breathed from a mask covering the mouth and nose, the expired air passing through a gas-meter. A sample was drawn slowly from the expired air line during each estimation. Owing to the likelihood that some overbreathing was taking place as a result of the thermal stress, metabolic rate was calculated from the oxygen consumption, assuming a value of 0·85 for the respiratory quotient.

Before entering the hot room, the subject rested, nude, for a period of half an hour in a room at

28° C, 40% r.h. and 50 ft./min air movement. The hot exposure lasted about an hour, but in some cases was terminated rather sooner than this on account of distress of the subject or observer. The duration in each case may be read from Fig. 2.

Determination of heat exchange constants

In order to provide values for the constants, k_e and k_{cr}, appearing in the equations being tested, a series of experiments was performed using a dummy instead of a living subject.

The dummy was jointed at the neck, shoulders, elbows, wrists, back, hips, knees and ankles, and could be arranged in the chair in a posture similar to that adopted by the subject. It was clothed all over in closely woven cotton under-wear, which was soaked in water. It was found that dripping continued from the buttocks and heels for so long that there was risk of some areas drying out before it had stopped. The drips were therefore allowed to fall into dishes containing liquid paraffin, which were placed on the balance pan. Evaporation could thus be measured although dripping was still occurring. An experiment was first carried out in order to discover for how long the dummy could be regarded as completely wet. It was set up in a fixed environment of low humidity and the weight recorded continuously. The rate of weight loss soon settled down to a steady level, which was maintained for about 40 min. After this time the rate of weight loss decreased, suggesting that some parts of the dummy were no longer completely wet. The duration of subsequent experiments was limited so that the total weight loss did not exceed half that which had occurred in this first experiment by the time the weight record began to deviate.

The wetted dummy was placed on the chair and the weight record started. When this had been straight for about 10 min, the surface temperature of the dummy was measured in the same regions and by the same technique as was used for the living subjects. The ambient humidity was then raised and weighing continued. The rising humidity produced a decrease in the rate of evaporation, which later partly recovered as the surface temperature rose. A second series of skin temperature observations was made when the weight loss had been steady for a further period of 10 min. The sequence of operations was continued until it became necessary to rewet the surface of the dummy. Ambient temperatures of 32, 36, and 40° C were used, and no effect of ambient temperature on the heat exchange constants was detectable. Humidities ranged from 10 mm Hg partial pressure to near saturation.

The evaporative constant, k_e, was found from the relation of water vapour pressure difference to the rate of water loss. The regression line for this relation had an insignificant intercept and a slope of $24{\cdot}40 \pm 0{\cdot}55$ (S.E.) g/hr. mm Hg. This is equivalent to $7{\cdot}81 \pm 0{\cdot}18$ (S.E.) kcal/m^2.hr. mm Hg, assuming a value of 0·576 kcal/g for the latent heat of evaporation of water and a surface area of 1·8 m^2.

The constant for heat loss by radiation and convection combined, k_{cr}, was found from the relation between the weight loss and the temperature difference between the dummy's surface and the air (and walls). Again the intercept was insignificant, the regression coefficient being $27{\cdot}72 \pm 2{\cdot}44$ g/hr. ° C. This is equivalent to $8{\cdot}87 \pm 0{\cdot}78$ kcal/m^2.hr. ° C.

Metabolic rate

Metabolic rate has been assumed to be constant for each subject and is taken as the mean of the measurements made on him during these experiments. The metabolic rate showed little change with time although there was a progressive increase in body temperature. It is probable that under steady body temperature conditions a fall in metabolic rate would have been observed because of the inactivity of the subject, and the absence of a significant change in practice may represent the fortuitous combination of these two opposing trends. It provides some justification for the assumption of a constant metabolic rate which is required in order to enter the equations. The rate used is not that found by dividing the total heat production by the Dubois surface area, since not all this area was available for heat loss. The arms were rested on some parts of the trunk, and allowance should be made for the area of scalp covered by hair. The heat-exchange constants determined using the dummy are related to the entire surface area and are expressed per unit area. As the effective area of the subject was less, and temperatures were not measured on apposed skin areas or on the scalp, appropriate adjustment may be made by expressing the metabolic rate as the heat production per unit skin area available for heat exchange. This neglects the probable difference between the relative proportions of the radiation and convection areas of the subject and dummy respectively. It has been assumed that both areas were reduced by 15%. It is probable that the error of neglecting this difference is small compared with that implicit in the guess of a 15% reduction in the convection-evaporation area. Its magnitude depends on the environment, and can be found for any given circumstances from equation (1).

RESULTS

The relation between mouth and mean skin temperatures is shown in Fig. 1, from which it will be seen that the slope appears to be unaffected by the severity of the environment within the limits used. The regression line through all points for D. B. has the slope 1·05 ± 0·03 (S.E.), and that for D. K. 1·06 ± 0·09. These coefficients do not differ significantly from unity, and provide justification for the omission from equation (2) of the weighting factors for skin and deep temperatures.

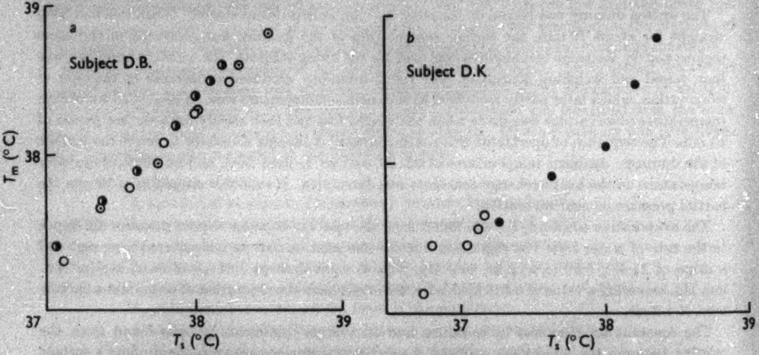

Fig. 1. Skin (T_s) and mouth (T_m) temperatures during exposure to severe environments. Calculated final skin temperatures (°C) for D.B. ◑ 39·3, ○ 39·7, ⊙ 40·1; for D.K. ○ 37·7, ● 39·5.

It proved impossible to check the final skin temperatures directly owing to the severity of the conditions required to ensure complete wetness. The skin temperature was therefore measured at intervals of about 10 min during the exposure, beginning when inspection showed the skin of all areas to be wet and dripping freely. Temperatures before this could not be used to test the equations since the necessary criteria were not satisfied.

For each experiment the final skin temperature was first calculated from equation (1). This figure is required in order to enter equation (2), so that if the temperature changes are appropriately described by equation (2) it is probable that the final skin temperature for that environment was properly predicted by equation (1). The natural logarithm of the difference between the observed skin temperature and its calculated final value, i.e. $(T_s^* - T_s)$, was next plotted against time. This is shown in Fig. 2. If equations (1) and (2) are correct descriptions of the situation, this plot should produce a straight line with a slope of $-\frac{A}{SW}(2{\cdot}6 \times k_e + k_{cr})$. Thus the slope should be independent of the ambient temperature and humidity, which will affect only the intercept. This intercept is of no significance in the present case since at time zero the skin was not completely wet. The test of equations (1) and (2) depends on the straightness of the lines and on whether their slopes have the predicted value. Linearity appears to be established in Fig. 2, and the lines are nearly parallel. Their slopes are presented in Table 1, as the time constants (negative inverse of slope). They have also been multiplied by $-\frac{SW}{A}$, to eliminate those quantities which depend on the characteristics of the subject. The expected value is therefore $(2{\cdot}6 \times k_e + k_{cr})$, or 29·2, and the observations provide values near to this. The mean is 29·83 ± 0·96 (S.E.), which does not differ significantly from 29·2.

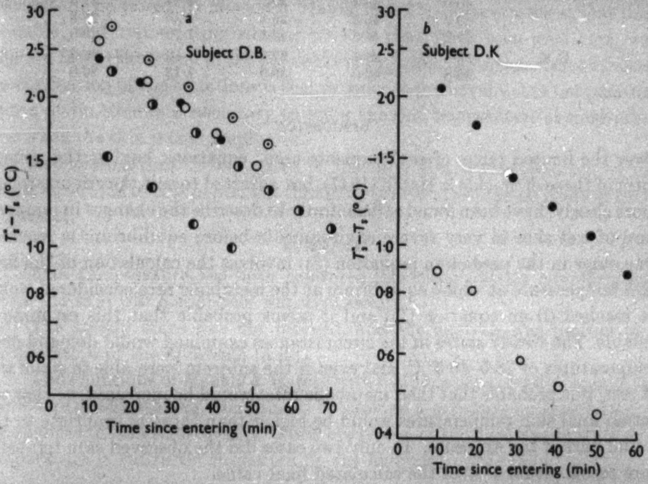

Fig. 2. The relation between time of exposure and difference between observed skin temperature and its calculated final value. Ordinates, temperature differences (log. scale); abscissae, time since entering hot environment. Final skin temperatures (° C) for D.B. ◐ 38·3, ◑ 39·3, ○ 39·7, ● 39·8, ⊙ 40·1; for D.K. ○ 37·7, ● 39·5.

TABLE 1. Ambient conditions, calculated final skin temperatures, observed time constants for skin temperature and estimates of $(2{\cdot}6 \times k_e + k_{cr})$ derived from the time constants. T_a ambient dry-bulb temperature, E_a ambient water vapour pressure, T_s^* final skin temperature calculated from equation (1) in the text. The time constant, K, is derived from the slope of the line plotted in Fig. 2. The last column shows the inverse of the time constant multiplied by $\frac{SW}{A}$ for the subject concerned. The expected value in this column is 29·2

Subject	T_a (° C)	E_a (mm Hg)	T_s^* (° C)	K	$\frac{SW}{AK}$
D.B.	41·3	38·5	38·3	1·24	29·1
	40·2	43·6	39·3	1·24	29·1
	40·0	45·4	39·7	1·14	31·6
	40·2	45·3	29·8	1·25	28·8
	39·9	46·8	40·1	1·20	29·9
D.K.	39·8	39·4	37·7	1·12	30·3
	39·8	46·0	39·5	1·13	30·0

DISCUSSION

Over the limited range of environments used, equations having the general form of those of Machle & Hatch (1947), but adjusted to suit the circumstances more closely, have been found satisfactorily to describe the changes in temperature of wet skin in very severe environments before equilibrium is reached. One stage in the prediction (equation (2)) involves the calculation of the final skin temperature at which equilibrium at the metabolic rate considered would be reached (from equation (1)) and it seems probable that this estimate is reliable. The steady states in the circumstances examined would demand deep temperatures of 38·5–40·5° C, and even if the subjects were able to stand this at rest, it is probable that their metabolic rates would be increased, so that the actual final skin temperatures would be higher than those appropriate to the initial part of the exposure. In only two cases did the observed skin temperature reach within 1° C of the calculated final value.

For the calculation of final skin temperature it is necessary to know the constants for heat exchange by convection and radiation and by evaporation. If wall temperature had differed from air temperature, the radiation and convection constants would have been required separately. In the present work the constants were determined experimentally using a dummy in the posture and location of the subject. Use of the same technique for skin-temperature measurements for the subject and dummy probably reduced the effect of such systematic errors as are inherent in the method used. The only differences between the dummy and the subject (for the present purpose) were in the metabolic heat production, heat capacity, skin texture, respiratory movements and details of topography and posture. The resulting vindication of the equations is thus limited to a demonstration of the differences due to metabolic rate and heat capacity and of the insignificance of other differences which were not allowed for in the formulae.

From a practical point of view the conclusions which may be drawn are that the fundamental theory on which the equations of Machle & Hatch are based is apparently sound, and the final skin temperatures predicted by their method are probably correct in the case of wet skin. The application of the formulae is dependent upon ability to convert measurements of air movement into heat exchange constants, and to express heat exchange by radiation accurately. For the description of the changes in skin temperature before equilibrium is reached it is also necessary to have knowledge of the distribution of stored heat. This appears to depend on the severity of the stress, since the distribution proposed by Machle & Hatch, based on observations at that time available, and representing smaller stresses than have been used here, was different from that found in the present investigation. A further factor limiting the application of the equations is that in order to predict the skin temperature after a given time it is necessary to know the skin temperature at some other time when the skin is completely wet.

It is concluded that the equations of Machle & Hatch are supported by the results described in this paper, but that little practical use can be made of them at present.

SUMMARY

1. Skin temperature has been measured in resting men in circumstances where the skin was completely wet with sweat.

2. The steady state was not reached, and the analysis was limited to a consideration of the approach to it.

3. The equations of Machle & Hatch, with modifications of detail to suit the particular circumstances, were found to provide a satisfactory description of the changes in skin temperature. Their prediction of the final steady state skin temperature is indirectly supported by the findings.

We are grateful to the Director-General of Medical Services, Royal Air Force, for permission to submit this work for publication.

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THE RELATION BETWEEN THE COEFFICIENTS FOR HEAT EXCHANGE BY CONVECTION AND BY EVAPORATION IN MAN

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The exchange of heat between an object and the surrounding air may be considered to take place across a boundary air layer whose thickness depends on the ambient air movement and on the shape, size and surface characteristics of the object. If water vapour is being exchanged between the object and the air, this diffusion must also take place through the boundary air layer, and it might be expected that the rates of exchange of heat and of water vapour should both be related in the same way to the thickness of the boundary air layer. Thus if a certain change in boundary air layer thickness doubled the heat exchange by convection for a given temperature difference, it might be supposed that the water-vapour exchange for a given vapour pressure difference would also be doubled. This proposition involves the assumption that the diffusivities of heat and of water vapour in air are the same, and it happens that they are in fact nearly equal. Discrepancies are to be expected when the temperature difference is great or when the vapour pressures considered are comparable with the atmospheric pressure, but these effects are likely to be small in most conditions encountered in 'hot climate' physiology (Jakob, 1949).

The assumption of a constant ratio between the coefficients for heat exchange by evaporation and by convection is implicit in Brunt's analysis of human heat balance (Brunt, 1947), and has been stated axiomatically in that of Woodcock, Powers & Breckenridge (1956), who give the ratio as 2·0, when the units used are kcal/m^2.hr.mm Hg and kcal/m^2.hr.°C respectively. Observations on three inert bodies, a thermometer bulb, a sphere of 6 in. diameter and a full-sized dummy man, have confirmed this value (Kerslake & Waddell, 1957). The present paper describes the results of an investigation of the ratio in human subjects.

METHODS

It is impossible to measure the convection coefficient, k_c, alone, since heat exchange by radiation is always present. The sum of the coefficients for convection and radiation exchange, $(k_c + k_r)$, can, however, be determined from consideration of heat balance. In circumstances where the dry-bulb temperature exceeds the skin temperature, the exchanges may be equated thus

$$E = C + R + M - S,$$

where E is the heat loss by evaporation, C the gain by convection, R the gain by radiation, M the metabolic heat production and S the rate of storage of heat in the body, positive if heat is stored. All these are expressed per unit area of skin. E is readily found by weighing, M can be measured and S estimated from temperature changes in the body. $(C + R)$ can then be found by difference. $(k_c + k_r)$ is calculated by dividing $(C + R)$ by the temperature difference between the air and the skin. It is, of course, necessary that the wall temperature shall equal the air temperature. The weakest link in the determination is the estimation of S, and the least inaccuracy is introduced when the value of S is small, that is when the subject is near the steady state.

For the determination of k_e it is essential that the skin shall be completely wet with sweat, and in order to ensure this it is necessary that the rate of sweat production shall greatly exceed the rate of evaporation. A factor of two is probably required here. It was found in practice very difficult to produce environments satisfying this requirement in which the steady state could be attained, and so the determinations of k_e and of $(k_c + k_r)$ were conducted separately in environments having the same temperature and air movement, but in which the humidity was varied to suit the determination being made. Variations in humidity do not affect the transfer of sensible heat under these conditions (Hill, Griffith & Flack, 1916; Burton, 1934).

Measurements. The subject reclined upon a nylon net supported on the platform of a recording balance. Dripping sweat was allowed to fall into trays containing liquid paraffin, which were supported on the balance pan. The recorded weight loss, when corrected for metabolic exchange, thus represented total evaporative water loss. Skin temperature was measured at the ankle (1 point), calf (2), thigh (3), trunk (5), arm (1), forearm (1), hand (1) and forehead (1) by means of a touch-on thermocouple calibrated during each experiment. Mean skin temperature was expressed as the arithmetic mean of the fifteen temperature readings, the distribution being approximately correct in terms of the areas of the different regions. Mouth temperature was measured with the same thermocouple. Air temperature and humidity were measured with an aspirating psychrometer upwind of the subject. Wall temperatures were assumed to be equal to air temperature. The balance was enclosed above, below and on two sides by a booth made of thin aluminium sheet. Behind the subject a system of louvres made of the same material allowed air to pass out while preventing the passage of radiation. In front of the subject was a narrow mesh wire screen, beyond which were air-straightening vanes also presumed to be at air temperature. All but these last surfaces were painted matt black, and it is likely that the total emissivity was near to unity. Air movement was not measured, but was approximately linear and horizontal, the subject facing into the wind.

Determination of k_e. Ambient conditions were approximately 48° C dry bulb and 40 mm Hg vapour pressure. The subject was exposed to the environment for about 20 min before observations were started. The skin temperatures were then measured and a record of weight loss taken for 15 min. At the end of this time the skin temperatures were again measured. The mean of all the skin temperatures was taken and the skin vapour pressure was assumed to be equal to saturated water-vapour pressure at this temperature. The rate of water loss by evaporation (respiratory water loss was neglected) was multiplied by 0·576 and divided by the subject's surface area to give the evaporative heat loss per unit area, and k_e was found by dividing this by the difference between skin vapour pressure and ambient vapour pressure. Other heat exchanges are immaterial to this determination, and although the skin temperature was rising the change in skin vapour pressure over the period of the observation was small.

Determination of (k_c+k_r). Ambient temperature was about 48° C with a low humidity (6–10 mm Hg). The subject was exposed to the environment for 40 min before observations were started. The skin and mouth temperatures were then measured and a record of weight loss taken for 20 min. At the end of this time the temperature measurements were repeated. Heat storage was calculated from the changes in skin and mouth temperature, allowing weightings of one third and two thirds respectively. The specific heat of the body was taken as 0·83 (Burton, 1935). Metabolic heat production was assumed to be 50 kcal/m^2.hr (see below). The total evaporative heat loss was found from the weight loss, and heat gain by convection and radiation was found by difference. This was divided by the temperature difference between the air (and walls) and the skin, to give (k_c+k_r).

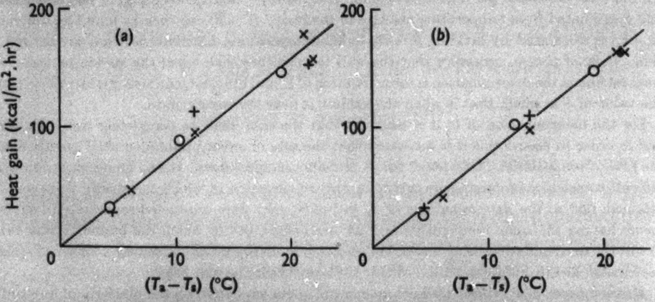

Fig. 1. The relation between heat gain by convection and radiation and temperature difference between air (and walls) and skin: *a*, using observed metabolic rates; *b*, assuming a metabolic rate of 50 kcal/m^2. hr. Air movement constant; humidity low.

It was desired to avoid the use of a face mask, which would have been required for the determination of metabolic rate, since mouth temperature was being measured. Determinations of (k_c+k_r) had previously been made at fixed air movement in another situation by the same technique, except that metabolic rate had been measured. Examination of the results showed that the assumption of a metabolic rate of 50 kcal/m^2.hr was without effect on the value derived and did not decrease the accuracy of the determination. The results are presented in Fig. 1, where temperature difference between air and skin is plotted against the calculated heat gain by convection and radiation (*a*) using the observed metabolic rates, and (*b*) assuming a value of 50 kcal/m^2.hr. On the basis of these observations it was considered justifiable to omit the measurement of metabolic rate in the present experiments.

RESULTS

Determinations of k_e and (k_c+k_r) have been made on two subjects at four different levels of air movement. The statement under examination is that the ratio of k_e to k_c is constant at all air movements and has the value 2·0. This may be tested by plotting k_e against (k_c+k_r), since k_r should be independent of air movement. The results are presented in this form in Fig. 2. The line drawn in this figure is the theoretical expectation, having a slope of 2·0 and an intercept equal to the calculated radiation constant. The calculation was carried out as follows. The radiation exchange between two black surfaces at skin and wall temperature respectively was worked out from the Stefan-Boltzmann relation, using the difference between the fourth powers of the two absolute temperatures. This amount of heat was then divided by the temperature difference to give the value of k_r for these conditions. Since the radiation area of the body is less than the DuBois area used for the expression of the heat exchanges, the calculated value of k_r was multiplied by a factor representing the ratio of the radiation area to the total area. This has been determined photographically by Guibert & Taylor (1951), who found the factor to be about 0·7 for the posture used. The value derived by this method for the temperatures used was 4·3. The observed points in Fig. 2 are scattered fairly evenly about the line, and it appears that the results are adequately described by it.

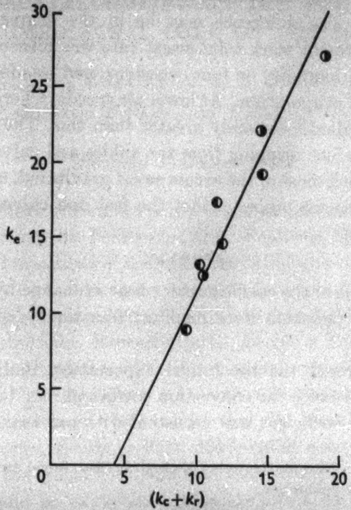

Fig. 2. The relation between the evaporation constant, k_e, and the sum of the convection and radiation constants, $(k_c + k_r)$. The line drawn shows the expected relation, and has a slope of 2·0 and an intercept of 4·3. ◐, subject D.B.; ◑, subject D.K.

It is concluded that over the physiological range of vapour pressures and for air temperatures between 35 and 48° C the evaporation coefficient may be taken as twice the convection coefficient, when mm Hg and °C respectively are used to define the gradients.

DISCUSSION

Although physical theory would lead one to expect a constant ratio between k_e and k_c over the physiological range of temperatures and vapour pressures, previous determinations have not given this result (Machle & Hatch, 1947). The findings described in the present paper, on the other hand, are consistent with expectations. The difference may lie in the degree of skin wetness achieved. In the present work total sweat rate was measured at the higher air speeds with high humidity on four occasions, and was found to be at least 2·3 times the rate of evaporation. At lower air speeds, where evaporation was less, the ratio was almost certainly greater than this. The sweat rates were great enough to produce dripping from the ankles and calves as well as from the buttocks, to which most of the excess sweat gravitated, and at lower ratios than those used it seems probable that the feet and calves would not have been completely wet.

SUMMARY

1. Determinations of the coefficients for heat exchange by evaporation and by convection and radiation were made at four air speeds on two human subjects at rest.

2. Results confirmed the theoretical expectation that the evaporation coefficient should be twice the convection coefficient.

3. The radiation coefficient was consistent with previous determinations.

We are grateful to the Director-General of Medical Services, Royal Air Force, for permission to submit this paper for publication.

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EFFECTS OF ACETYLCHOLINE ON THE ACTIVITY OF THE LUMBOSACRAL CORD OF THE CAT

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Acetylcholine has been known, for many years, to influence the activity of the spinal cord (see review by Feldberg, 1950). Feldberg, Gray & Perry (1953) made close arterial injections of acetylcholine (ACh) into the basilar arteries of cats anaesthetized with chloralose; recording from the first cervical ventral roots they observed increased numbers of impulses and increases in the reflexes and root potentials. Recently Curtis, Eccles & Eccles (1957) have injected ACh into the circulation of the lumbosacral cord of cats anaesthetized with pentobarbital and observed a reduction in the amplitude of certain reflexes. They argued from their results that the known effect of ACh on Renshaw cells (Eccles, Eccles & Fatt, 1956) could account for the over-all action of ACh on the spinal cord, and that the excitatory effects observed by Feldberg *et al.* could be explained by the excitation by ACh of afferent receptors (see review by Gray & Diamond 1957). This paper presents experimental results that indicate that ACh must have actions in the spinal cord in addition to those on the Renshaw cells.

METHODS

Preparations. Most experiments were performed on cats whose cords had been divided in the high cervical region and brains destroyed under ether anaesthesia. Satisfactory preparations showed vigorous tendon and withdrawal reflexes. Chloralose anaesthesia, 100 mg/kg, without section of the cord, was used in a number of preparations. Most animals received 1 mg atropine intravenously.

Recording. A laminectomy was performed to expose the low lumbar and sacral spinal cord and was extended caudally for a sufficient distance to obtain the full length of the first sacral ventral root. Records of activity were obtained from ventral rootlets with platinum wire electrodes and from the dorsal horn with glass capillary electrodes of 1–5μ tip diameter filled with 10% NaCl solution. The potentials were amplified with a system having a high input impedance and variable frequency response.

Stimulation. Dorsal roots, the sciatic nerve in the thigh or the sural nerve in the popliteal space were stimulated with short (< 0·5 msec) pulses from a low impedance source isolated from earth.

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Injections. A number of techniques were used, all of which were intended to allow the ACh to reach the cord and as few other tissues as possible, as rapidly as possible and with a minimal mixing with blood. Of these, three should be mentioned: (1) retrograde cannulation of one internal iliac with tying or snaring the aorta above the seventh lumbar pair and tying all branches below this except other dorsal pairs, the medial sacral artery and in most experiments on the side used for nerve stimulation the external iliac, which was snared; (2) cannulation of one renal artery with the tying of all branches of the admonal aorta except the dorsal pairs and on one side the external iliac, which was snared; or (3) cannulation of a lumbar artery. In exposing the spinal cord all arteries that were seen to supply muscles were tied. In all experiments the cannula consisted of a short piece of stainless steel tube connected to a polythene tube which was passed out of the abdomen. Heparin was used in the cannula.

Solutions. All injections were made with a solution containing (g/l.) NaCl 9·0, KCl 0·42, $NaHCO_3$ 0·15, $CaCl_2$ 0·24. ACh (chloride) was dissolved in this solution immediately before use. Further details of the techniques and procedures have already been published (Fernandez de Molina & Gray, 1957).

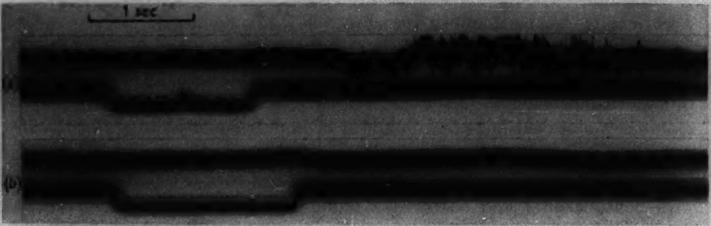

Fig. 1. Activity in dorsal horn and ventral root evoked by ACh. Top traces, records from electrode in dorsal horn of S1. Bottom traces, records from ventral rootlet of S1, and injection signal (approx. only). Time 1 sec. (*a*) injection of ACh, 1 ml. of 10^{-4} mg/ml. (*b*) control, dorsal roots of S1 cut.

RESULTS

Ventral root

Discharge excited by acetylcholine. Spinal preparations that were in good condition responded to injections of ACh (1 ml. of 10^{-4} g/ml.) with discharges of impulses in the ventral roots (Figs. 1–3); this dose was that normally used, but 1 ml. of 10^{-5} g/ml. was shown to be effective. Such discharges were not, however, found regularly in anaesthetized animals; they were only seen in four out of thirteen preparations and these instances were all doubtful. These discharges were seen both in the absence of stimulation (Fig. 1) and when reflexes were excited at regular intervals (Figs. 2, 3). Reflex excitation of this kind sometimes altered the pattern of the discharge. In one experiment ACh caused a discharge which started immediately after each stimulus and then decreased in frequency. Another experiment (Fig. 3) showed the reverse phenomenon; before the injection and at the end of the record the impulses occurred mainly in the early part of the interval, while immediately after the injection there was a pause that followed the stimulus and the rest of the interval was occupied by a considerable number of impulses.

Ventral root discharges in response to ACh do not appear to be due to an increased afferent activity resulting from excitation of receptors by ACh. The effects of ACh on receptors can be blocked by curarine and by hexamethonium (see Gray & Diamond, 1957), but the discharge still occurred after 22 mg/kg of tubocurarine and after 10 mg/kg of hexamethonium. Such discharges were seen after wide deafferentation (L5 to end) in three experiments. Fig. 2 was recorded after the injection of hexamethonium but before deafferentation, while Fig. 3 was recorded from the same preparation after crushing all dorsal roots below and including L5; there is little difference in the discharges of impulses recorded from the ventral roots.

Reflexes. Our results agree with Curtis *et al.* (1957) in that the usual effect of ACh was to reduce the amplitude of both monosynaptic and polysynaptic reflexes. Increases in reflexes after deafferentation have however been seen twice, one occasion being illustrated in Fig. 3. In this experiment the dorsal root of S1 was stimulated and it can be seen that the injection has caused an increase in the monosynaptic spike while reducing the polysynaptic response. Sometimes an increase in the reflex size has been seen after an initial decrease.

Dorsal horn activity

Discharges elicited by acetylcholine. Increased activity in the dorsal horn was recorded after injections of ACh. No attempt has been made to plot the distribution of this activity, all records having been taken when the electrode had been placed to record activity set up by stimulation of cutaneous nerves (Coombs, Curtis & Landgren, 1956; Fernandez de Molina & Gray, 1957). The activity, which can be seen in Figs. 1–4, consists of spike activity and slower waves. The latter are seen with little spike activity in Fig. 4 and with many spikes superimposed in Fig. 1. This activity follows an injection of ACh after deafferentation or hexamethonium (Figs. 2, 3).

Potentials elicited by stimulation of cutaneous afferents. Fernandez de Molina & Gray (1957) divided the dorsal horn activity that results from cutaneous nerve stimulation into three phases: phase I occurs within 1 msec of the afferent volley and in the region of the internal basilar nucleus; phase II is maximal at 1–3 msec and found in the ventrolateral region of the dorsal horn; phase III is maximal 8–12 msec after the afferent volley. The effect of ACh on all three phases of potential change has been studied. Investigations of phase I did not lead to any definite conclusions, as this response is found in a region in which the potential gradients are large. Control injections, apparently by moving the cord in relation to the electrode, caused changes in the amplitude of this phase. Phase II, which was investigated most frequently, was consistently reduced in amplitude by ACh. This is well shown in Fig. 4. Each

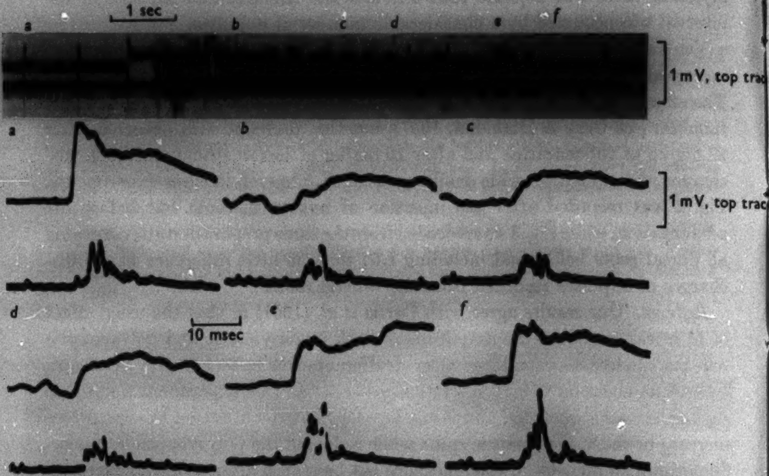

Fig. 2. Effect of ACh, 1 ml. of 10^{-4} g/ml., on activity in dorsal horn and ventral root of a rhythmically stimulated preparation. Top record on slow time scale (1 sec); lower records faster samples (time 10 msec) at times indicated on top record. In all records; top beam from dorsal horn and injection signal (approx. only); bottom beam from ventral root. Animal had received hexamethonium 10 mg/kg. Sural stimulation. (Ventral root spikes retouched in top record.)

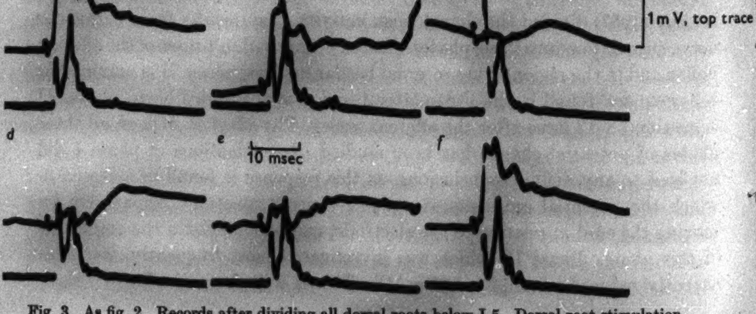

Fig. 3. As fig. 2. Records after dividing all dorsal roots below L5. Dorsal root stimulation. (Ventral root spikes retouched in top record.)

record in the lower part of the figure is a photograph of nine superimposed traces, while the record at the top shows the time course of the same events. The potential recorded is almost entirely a phase II potential, though in this instance it was obtained by stimulation of the dorsal root rather than a pure cutaneous nerve. An injection of ACh reduced phase II to about a third of its original value. Another experiment is illustrated in Fig. 2 but in this the sural

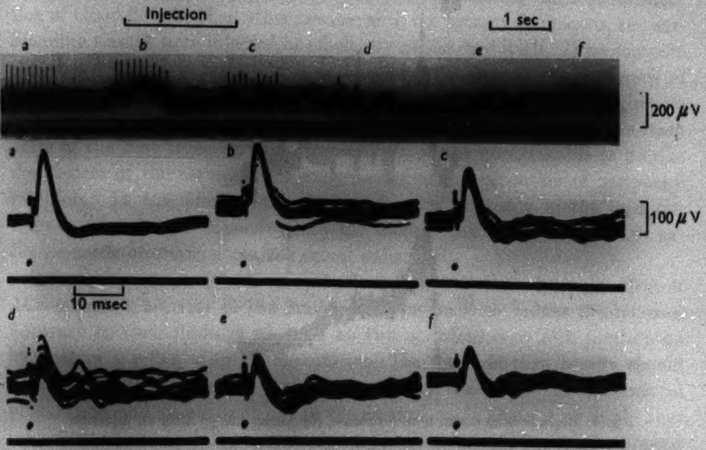

Fig. 4. Effect of ACh, 1 ml. of 10^{-4} g/ml., on phase II of the dorsal horn activity. Top record on slow time scale (1 sec); lower records, photographs of superimposed traces (time 10 msec) of each group of stimuli. In all records; top beam, dorsal horn potential; bottom beam, stimulus. Dorsal roots of S1 cut. Dorsal root stimulation.

nerve was stimulated. The ACh injection virtually abolished phase II. These changes in the activity of the dorsal horn do not appear to be related to changes in afferent inflow. The records shown in Fig. 2 were obtained after the animal had received 10 mg/kg of hexamethonium, which would be expected to block the stimulating action of ACh on receptors. Furthermore, the same results appear in Fig. 3, which was obtained from the same preparation after all dorsal roots caudal to L5 had been divided. In one experiment the changes in phase III were investigated and a considerable reduction in amplitude observed.

Spike amplitude in the records in the lower parts of Figs. 2–4 was considerably attenuated by reducing the high-frequency response of the amplifier. Fig. 5 was recorded with an adequate high-frequency response and it can be seen that there is an increase in the spike activity associated with both phases II and III.

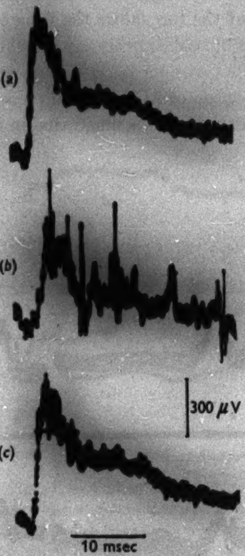

Fig. 5. Records from dorsal horn (*a*) before, (*b*) during and (*c*) after an injection of 1 ml. of 10^{-4} g/ml. ACh.

DISCUSSION

Activity in the dorsal horn and in the ventral root was induced by ACh; that this occurred in preparations in which an increased afferent inflow had been prevented indicates that the activity was set up by a central action of ACh. The behaviour of the lumbosacral cord to injections into the aorta was however markedly different from that of the cervical cord to injections into the basilar artery (reported by Feldberg *et al.* 1953). In the anaesthetized animals of the present series the discharge was usually absent, or if present was very slight; in the experiments of Feldberg *et al.* (1953) on the cervical cord, however, massive discharges were normally observed in animals anaesthetized with chloralose, though such impulse activity could be much reduced or abolished with barbiturates. The explanation of this difference would seem to be that with basilar artery injections the solution goes directly into the vascular supply of the cord; during the injection the segments under consideration become momentarily blood free and in consequence a high concentration gradient between the vascular bed and the tissue is built up very rapidly. It is well known that in order to demonstrate the excitant action of ACh a rapid rise of concentration may be necessary (Brown, Dale & Feldberg, 1936); it is more likely to be important in the central nervous system where the problem of the 'blood-brain barrier' arises. In the present experiments none of the injection techniques was able to produce more than a local blanching of some vessels in the cord and usually no obvious blanching was seen during an injection. Another important difference is that in the cervical cord preparation only such part of the injection as flowed sufficiently far against the normal direction of blood flow reached other tissues; with the present techniques, the surrounding tissues were injected as efficiently as the cord itself. If we compare the almost complete failure of injections into the anaesthetized animals of this series even when not deafferented, with the success of the experiments described by Feldberg *et al.* (1953) on a preparation which had had the main parts of the first three cervical nerves cut, it seems unreasonable to ascribe the success of the cervical cord experiments to the excitation of peripheral receptors. In the present experiments the low efficiency of the injection technique has been compensated by the ability to use the much more excitable cords of unanaesthetized spinal animals.

Our results agree with those of Curtis *et al.* (1957) that the main effect of ACh on reflex activity in the lumbosacral region is to reduce the reflexes. Contrary to their findings we have on two occasions seen ACh produce an increase in a reflex after deafferentation. The question then is why should ACh have a predominantly depressant effect on reflexes in the lumbosacral preparation and an excitant one in the cervical cord. There is no doubt that inhibitory effects of ACh are to be found (Eccles *et al.* 1956), and the present results confirm the finding of Feldberg *et al.* that excitatory ones also occur; which of the two predominates at any time depends on the organization and state of activity of the particular part of the cord. There are certainly differences between the organization in the lumbo-sacral cord and in the cervical cord; it would be interesting to know if antidromic (Renshaw) inhibition was as marked in the cervical region as in the lumbosacral. An important point to emphasize is the variability of these phenomena; for example, one spinal preparation gave an increase in reflexes with ACh when they were excited by sural stimulation but a decrease when they were excited by dorsal root stimulation; similarly, the activity that follows immediately after a reflex response has been both increased and decreased by ACh.

The potential changes in the dorsal horn that can be elicited by sural stimulation show a decrease in amplitude during an injection of ACh. These changes can hardly be due to activation of Renshaw cells. It is impossible to be certain about the cause of this decline in amplitude. It could be due to the excitation of inhibitory pathways, which converge on those stimulated electrically, but it could also be due to the fact that these artificially stimulated potentials are depressed when they occur at the time of the intense asynchronous activity that results from the ACh injection. During such intense asynchronous activity many cells are refractory at any one time so that a synchronous response to stimulation becomes smaller.

SUMMARY

1. Techniques for injecting solutions into the circulation of the lumbosacral cord have been used to investigate the action of acetylcholine on its activity.

2. In spinal animals acetylcholine itself excited a discharge of impulses in the ventral root. This is not due to an increased afferent inflow. This response was seldom seen in anaesthetized animals and if it was present it was very small.

3. Reflexes, monosynaptic and polysynaptic, were normally reduced in amplitude (Curtis *et al.* 1957). Increases have, however, been seen in deafferented preparations.

4. Acetylcholine excited activity in the dorsal horn and caused a reduction in amplitude of the potential changes that result from cutaneous nerve stimulation.

5. It is argued that acetylcholine has an excitant action on the spinal cord. Its over-all effects cannot be entirely explained by its known action on the Renshaw cells.

Our thanks are due to the Consejo Superior de Investigaciones Cientificas for a grant to one of us (A. Fernandez de Molina).

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ELECTROLYTE EXCRETION IN DIURETIC AND NON-DIURETIC DOGS

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A number of observations made in man have shown that during water diuresis there is a reduction in the rate of loss of Na and Cl (Priestley, 1921; Adolph & Ericson, 1926; Eggleton, 1943; Crutchfield & Wood, 1948). Individually exceptional responses occurred (Eggleton, 1943), and the previous history of the water intake had to be considered (Crutchfield & Wood, 1948). Information regarding electrolyte excretion in dogs is limited. Karvonen & Leppänen (1952) found a significant positive correlation between the rate of urine flow and the rate of Na excretion. A similar positive correlation between water and K and NH_4 excretion was found by Friberg, Karvonen & Leppänen (1950). The results of these workers are the opposite of those found in this laboratory, where the dogs showed a secretory pattern similar to that recorded for man. Since it is of importance to know whether there is a direct relationship between Na and water excretion it seemed advisable to record the results of the large number of observations made in this laboratory.

METHODS

The observations were made on normal anoestrous bitches weighing from 8 to 20 kg. The dogs had been prepared, at operation, for easy urethral catheterization by slitting open the dorsal perineum and stitching together the cut edges of the skin and mucous membrane. On days on which a dog was to be used it was given 250 ml. water at noon (the one 8 kg dog was given 200 ml.), then at 2.15–2.30 p.m. of the same day water diuresis was induced by the oral administration of 200–350 ml. water, according to the size of the dog. Observations were made with the dog lying down, and urine was collected from a self-retaining catheter passed into the bladder. In order to maintain a slight negative pressure the end of the collecting tube was always 5–10 cm below the table on which the dog was lying. The length of the connexion between the collecting cylinder and the catheter as it emerged from the vagina was kept as short as possible, and the dead space of the urine collection system was minimized by emptying the external part of the system with air blown through it from a side limb near the vagina. Urine samples were collected for 5–10 min into dry glassware which had been carefully washed in Na- and K-free water. For observations on the 'resting rate' of urine flow the dogs were prepared as described above except that the afternoon dose of water was omitted. Thus, on these occasions the dogs were hydrated but not in a state of diuresis. The dogs' diet consisted of milk, bread, gravy and dried meat, the last being replaced sometimes by rabbit meat. A constant measure of salt was added daily to the common stew-pot.

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Estimations of Na and K were made on an EEL flame photometer, and Cl was estimated by the method of Prout-Winter as described by Cole (1919).

RESULTS

A total of 240 water diuresis curves were made on nine dogs. Forty of the observations were controls. In the remaining two hundred observations 38–60 min after the ingestion of water some experimental procedure was begun. It was only in the early part of diuresis that variations in electrolyte excretion occurred and the experimental curves covered this period; thus the experimental curves may be considered together with the controls. On all occasions the rate of Na and K excretion was measured, and in over half of the observations the urinary Cl was also estimated. Six of the nine dogs consistently showed a fall in the rate of electrolyte excretion during the course of water diuresis (Fig. 1). Table 1 shows the results of six control observations in one dog; the same pattern of electrolyte excretion was seen even though the initial rate of salt excretion and that at the peak of diuresis varied over a fairly wide range. In two of the nine dogs the excretory pattern was usually as shown in Fig. 1, but in three out of twenty-one observations made on one dog and in four out of ten observations made on the other there occurred an increase in Na and Cl excretion, with a rising or steady rate of K excretion during the first 30–35 min of diuresis (Fig. 2). One of the nine dogs showed the pattern of excretion illustrated in Fig. 3 in two out of sixteen observations; after an early rise, the rate of Na loss fell steadily, but the originally low K excretion rose for 50 min before it declined. The few exceptional curves were seen when the excretion rates of Na, K and Cl were within limits which, on other days, were associated with normal curves, and no reason can be suggested for the different pattern of response. The pattern of Na excretion during diuresis shown in Fig. 2 has once been seen in a dog with well-developed diabetes insipidus.

An early fall in the rate of salt loss was also seen when the dogs were non-diuretic, but had been hydrated as usual 2–2½ hr previously. A total of 180 observations were made at this 'resting rate' of flow, of which 32 were controls. Fig. 4 is typical of all the results, and it can be seen that a steady state of water and electrolyte excretion was reached after 30–40 min collection, and that this was not disturbed by the intravenous injection of 1·0 ml. 0·9% NaCl solution.

In the observations made at a resting rate of urine flow there was always a steady reduction in the rate of electrolyte loss during the first 30–40 min. This suggests that both at rest and in diuresis the initially higher rates of electrolyte excretion might be due, in part, to some disturbance or excitement.

On three occasions only in two dogs, after an hour of observations made at rest, water was given and the first hour of diuresis observed. A longer period was not possible as the dogs became restless. In one dog on two occasions giving water, which involved raising the dog's head and inserting the stomach tube, caused no significant increase in the excretion rate of electrolytes. In the other dog in the first sample after water administration, the Na loss increased from 55 to 90 μequiv/min, K from 29 to 35 μequiv/min and Cl from 116 to 143 μequiv/min. Twenty minutes later K and Cl loss, and 30 min later Na loss, had returned to the values obtaining before water was given, despite

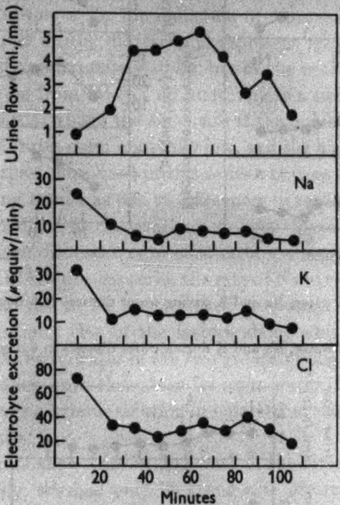

Fig. 1. The excretion of water, Na, K and Cl during water diuresis in a dog. At zero time, 350 ml. water by mouth.

TABLE 1. Rate of excretion of Na, K and Cl at the beginning and at the peak of water diuresis in one dog. Diuresis induced by oral administration of 350 ml. water

Max. rate urine flow (ml./min)	Sodium (μequiv/min) Initially	Sodium (μequiv/min) At peak diuresis	Potassium (μequiv/min) Initially	Potassium (μequiv/min) At peak diuresis	Chloride (μequiv/min) Initially	Chloride (μequiv/min) At peak diuresis
6·0	76	37	27	18	107	22
5·6	51	15	25	29	51	14
5·3	37	10	12	11	94	13
4·8	77	38	47	22	134	43
4·0	13	9	15	8	25	12
5·2*	120	80	70	18	200	80

* On this day the dog was hydrated in the morning with 0·9% NaCl solution, but was given the usual 350 ml. water in the afternoon.

a continuing rise in rate of urine flow. Possibly, then, the high values for Na, K and Cl excretion seen during the early part of all observations were partly related to the excitement of being brought from the kennels to the laboratory, a journey during which all the dogs tended to be playful.

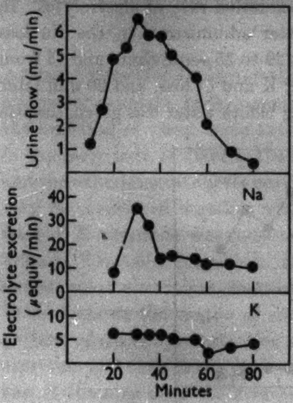

Fig. 2

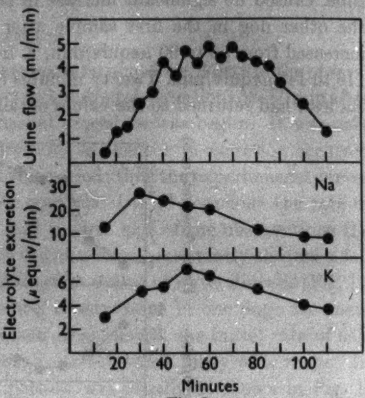

Fig. 3

Fig. 2. The excretion of water, Na and K during water diuresis in a dog. At zero time, 350 ml. water by mouth.

Fig. 3. The excretion of water, Na and K during water diuresis in a dog. At zero time, 350 ml. water by mouth.

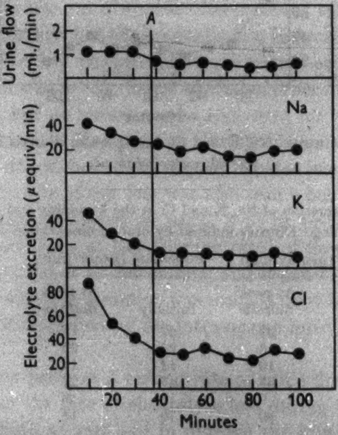

Fig. 4. The excretion of water, Na, K and Cl in non-diuretic dog. 250 ml. water by mouth 2·5 hr previously. At *A*, an intravenous injection of 1 ml. 0·9% NaCl solution.

DISCUSSION

It is clear from the above results that, with the routine used here, there was nearly always a steady decline in the excretion rate of Na, K and Cl during the course of a water diuresis. No positive correlation was ever seen between the loss of water and electrolytes; at the height of diuresis closely similar rates of urine flow were associated with widely varying rates of electrolyte loss (Table 1). These results are diametrically opposed to those of Karvonen & Leppänen (1952). The cause of the difference may lie in the techniques used, but this is difficult to judge since these authors do not say how much water was given for hydration or for the induction of diuresis, but only that their results were obtained during diuresis and represent spontaneous variations. Their dogs weighed approximately 10 kg, but at the peak of diuresis excreted urine at rates varying from 1·5 to 7 ml./min. This is a surprisingly wide range of variation for dogs of about the same size if the conditions were constant. Moreover, the lower rates seem unusually low, and the highest rate was barely attained by the largest dogs used in this series. It does not seem possible to explain the opposing results as due to differences in rate of Na excretion. The Finnish workers made their observations on very dilute urines in which the Na loss at the peak of diuresis was in most instances less than 10 μequiv/min. On some occasions in the present series the rate of Na excretion was below this initially, and yet no rise in Na loss occurred *pari passu* with the increase in rate of water excretion. One of the factors about which no information is available is the total body water in the two groups of dogs. It is possible that Karvonen & Leppänen gave their dogs far more water than was received by ours and that an over-all tissue dilution or enlarged extracellular fluid volume influenced the results.

The present results show that in dogs and in the circumstances described Na is not invariably 'washed out' by rising rate of urine flow, nor, within limits, is the maximum rate of urine flow in diuresis directly related to the Na loss. In fact, from the results of Anslow & Wesson (1955) the reverse might be expected. They found that in dogs an intravenous infusion of vasopressin caused an increase in the excretion rate of Na and Cl, which fell when the infusion ceased, and therefore in water diuresis a reduction in Na and Cl loss should be seen. In the present series of experiments this reduction occurred.

SUMMARY

1. Normal conscious dogs were observed during water diuresis and when non-diuretic, and the rates of loss of water, Na, K and Cl were noted.

2. During water diuresis, with few exceptions, there was a decline in the rate of excretion of electrolytes. When non-diuretic, and once the initial phase of excitement was ended, the dogs excreted electrolytes and water at a steady rate for a period of 2 hr at least.

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THE DISTRIBUTION OF THE CATECHOL AMINES IN HOMOGENATES OF THE BOVINE ADRENAL MEDULLA

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Three sympathomimetic amines have been found in the bovine adrenal medulla: adrenaline, noradrenaline and dopamine. Dopamine does not appear to accumulate in species other than the sheep (Goodall, 1951) and the ox (Shepherd & West, 1953); however, work on the formation of adrenaline and noradrenaline as well as the study of the distribution of the enzyme dopa-decarboxylase, which forms dopamine, make it likely that dopamine occurs also in other vertebrate species as the immediate precursor of noradrenaline (see Blaschko, 1957). No other function has been found for this amine, which is less active as a pressor agent than noradrenaline or adrenaline. Large amounts of dopamine occur in the sympathetic nerves of the ox (Schümann, 1956). In man dopamine is normally present in the urine (Holtz, Credner & Koepp, 1942; von Euler & Hellner, 1951); it also occurs in tumours of chromaffin tissue (Weil-Malherbe, 1956; McMillan, 1956).

When the localization of adrenaline and noradrenaline in cytoplasmic granules of the chromaffin tissue was first demonstrated, attempts were made to determine, by differential centrifugation, whether the distribution of adrenaline differed from that of noradrenaline. No such difference could be observed: both amines were sedimented in the granule fraction (Hagen, 1954). Similar observations were reported by Hillarp & Nilson (1954*a*).

In earlier experiments from this laboratory, when sediments of cytoplasmic granules were obtained from homogenates of bovine adrenal medulla in isotonic sucrose, a 'top' and a 'bottom' layer could be distinguished in these sediments (Blaschko, Hagen & Welch, 1955), but no investigation of the distribution of the two main catechol amines in these layers was made. More recently, a further fractionation of the 'large granule' fraction was achieved by centrifuging suspensions of large granules over strongly hypertonic sucrose solutions; it was possible to find concentrations of sucrose in which only some of the granules formed a sediment, whereas others were retained in the upper portions of the centrifuge tube (Blaschko, Born, D'Iorio & Eade, 1956).

In the present paper experiments are described in which the noradrenaline:adrenaline ratio of various granule fractions was determined. Observations on the distribution of dopamine in homogenates of bovine adrenal medulla will also be reported.

METHODS

Homogenates of bovine adrenal medulla in 0·3 M ('isotonic') sucrose were prepared; after removal of unbroken cells, coarse cell debris and nuclei by low-speed centrifugation (Eade, 1957), the supernatant obtained was centrifuged at 11,000 *g* for 20 min. The sediment from this centrifugation, the so-called large-granule fraction, was used as the starting material for further centrifugations.

The procedure for the collection of the 'top' and 'bottom' layers was the same as that used by Blaschko *et al.* (1956): the 'top' layer was the upper portion of the sedimented large-granule fraction; it was removed with a Pasteur pipette. The 'bottom' layer then remained in the centrifuge tube.

When hypertonic sucrose solutions were used, centrifugation was carried out in the horizontal rotor SW–39 L of the Spinco preparative ultracentrifuge. In all experiments the tubes contained 3·0 ml. of hypertonic sucrose solution of different molarities, and the large-granule fraction, suspended in isotonic sucrose, was layered over the hypertonic sucrose solutions. The tubes were centrifuged for 60 min at 142,000 *g* and the fractions obtained were collected with a Pasteur pipette.

For the determination of adrenaline and noradrenaline, N-HCl was added to the fractions; they were stored at about −12° C. For the determination of the total catechol amines the method of von Euler & Hamberg (1949) was used at pH 6·0, except that a sodium phosphate buffer (Clark, 1928) was substituted for the acetate buffer used by the Swedish authors.

On the basis of their total catechol amine content the fractions were diluted with 0·01 N-HCl–ethanol to give a final concentration of 200–250 μg of catechol amine in 1·0 ml. The proteins, which were precipitated by this procedure, were removed by centrifugation to give a clear supernatant fluid. The alcoholic extracts (usually 0·1 ml.) were applied to acid-washed Whatman No. 1 filter paper which had been treated with ascorbic acid; the papers were chromatographed in phenol–HCl (15% 0·01 N-HCl, w/v) for 18–20 hr at 26° C, under N_2, by a modification of the method described by Crawford & Outschoorn (1951). Each extract was applied in two positions, one of which served to locate the spots due to adrenaline and noradrenaline, while the other was used for the elution of the adrenaline and noradrenaline which had separated. Excess solvent was removed from the paper with benzene and the strips for elution were extracted in a micro-Soxhlet extractor four times with ether to remove traces of phenol. For the elution 0·01 N-HCl in isotonic saline, containing 1 mg ascorbic acid in 100 ml., was used.

The assay of the adrenaline (Ad) and noradrenaline (NAd) content of the eluates on the rat's blood pressure was carried out on pithed animals as described by Holzbauer & Vogt (1956).

Adenosinetriphosphate (ATP) was determined by the firefly luminescence method (Blaschko *et al.* 1956).

RESULTS

Adrenaline and noradrenaline in 'top' and 'bottom' layers

In three experiments the NAd:Ad ratio was determined in the top and bottom layers. The results are given in Table 1. The distribution of the two catechol amines in the two layers was very similar.

Distribution of adrenaline and noradrenaline in hypertonic sucrose solutions. When the large-granule fraction was centrifuged over hypertonic sucrose solutions, the sedimentation of the granules differed according to concentration of the sucrose solution. This is illustrated in Pl. 1, fig. 1, taken from an experiment in which the molarities of the sucrose solutions in the three tubes of the ultracentrifuge were 1·0, 1·5 and 2·0 M respectively; these molarities are those used by Blaschko *et al.* (1956). After centrifugation for 60 min at 142,000 *g*, the layer of isotonic sucrose at the top of the tube had been entirely cleared of opaque material. With 1·0 M sucrose in the lower part, very little granular material was retained in the boundary layer between the two solutions; the hypertonic sucrose solution was slightly opaque, but most of the particles had sedimented at the bottom of the tube. The figure shows that with increasing hypertonicity the amount of material retained in the boundary layer increased, whereas the amount of sediment decreased. Also, much more opaque material was retained suspended in the hypertonic sucrose layer.

TABLE 1. Bovine adrenal medulla. Ratio NAd:Ad in top and bottom layers

Expt. no.	Top layer	Bottom layer
1	0·41	0·46
2	0·38	0·38
3	0·67	0·70

TABLE 2. Recovery of catechol amines and NAd:Ad ratios in the sediment after centrifugation over hypertonic sucrose

Hypertonic sucrose (M)	Catechol amines found in the sediment* (%)	Ratio NAd:Ad†
1·6	65	0·44
1·8	28	0·56
2·0	13	1·41

* As percentage of total. † The ratio NAd:Ad in the large-granule suspension was 0·40.

From earlier experiments (Blaschko *et al.* 1956) it was known that in 1·5 M sucrose most of the catechol amine penetrated below the boundary between the isotonic and hypertonic sucrose, whereas in 2·0 M sucrose a large proportion of amine was retained in the boundary layer. It was therefore decided to carry out experiments using sucrose solutions of 1·6, 1·8 and 2·0 M concentrations, and to determine the total catechol amine content as well as the NAd:Ad ratio in the large-granule fraction, which served as the starting material for the ultracentrifugation, and in the three sediments obtained.

The results of this experiment are given in Table 2. In 1·6 M sucrose, approximately two-thirds of the total catechol amines was sedimented; in this sediment the ratio NAd:Ad was not significantly different from that in the large-granule fraction from which the sediment was derived. In 1·8 M sucrose, the NAd:Ad ratio of the sediment was a little higher. In 2·0 M sucrose, where only about one-eighth of the total catechol amine had sedimented, the NAd:Ad ratio had risen to 1·41. In other words, under these conditions noradrenaline had been preferentially sedimented.

Eight centrifugations were carried out in which 2·0 M sucrose was used as the hypertonic sucrose solution; the NAd:Ad ratio was determined in the large-granule fraction, in the sediment and in the boundary layer. The results obtained in these experiments are shown in Table 3. In each experiment the ratio NAd:Ad in the sediment was higher than in the initial suspension of large granules, whereas in the boundary layer the ratio was lower. In each experiment the total catechol amine in the three fractions was also determined. It could thus be calculated that of the total NAd 34·6 (±4·4)% was recovered in the sediment, whereas a mean of 14·7 (±2·7)% of the Ad was sedimented. On the other hand, 41·2 (±4·4)% of the NAd had been retained in the boundary layer, whereas 61·0 (±4·5)% of the Ad was found in this layer.

TABLE 3. NAd:Ad ratio in the large-granule suspensions and in fractions obtained after high-speed centrifugation of these suspensions over 2·0 M sucrose

	NAd:Ad ratio of		
Expt. no.	Large granules	Boundary layer	Sediment
1	0·40	0·34	1·41
2	0·70	0·28	2·84
3	0·32	0·20	0·75
4	0·58	0·40	1·20
5	0·35	0·19	0·75
6	0·33	0·23	0·62
7	0·36	0·31	0·75
8	0·24	0·19	0·63

The difference in the distribution of NAd and Ad in the large-granule fraction, in the sediment and in the boundary layer could be demonstrated when chromatograms were sprayed with the alkaline potassium ferricyanide reagent (James, 1948). In Fig. 2 the chromatogram was obtained from extracts in 0·01 N-HCl–ethanol, each extract containing 10 μg of total catechol amine as determined by the method of von Euler & Hamberg (1949). The chromatogram was run for 18 hr at 26° C in phenol–HCl; it was sprayed with the reagent and dried; a Kodak-contura contact print was prepared; Pl. 1, fig. 2, shows a photograph of this print. It can be seen that in the sediment the spot due to NAd was larger than in the large-granule fraction, whereas in the boundary layer this spot was less conspicuous. Conversely, the spot for Ad in the sediment was smaller than in both the original fraction and in the boundary layer.

In two of the eight experiments shown in Table 3, determinations were made not only of the catechol amines, but also of the ATP content; the results obtained are shown in Table 4. The molar ratio, 'Ad:ATP', of the large-granule fraction was very similar in the two experiments, 5·5 and 5·7 respectively. There was no significant difference in the molar ratio 'Ad:ATP' in the two fractions derived from the large granules.

The determination of the NAd:Ad ratio by the method of Crawford & Outschoorn (1951) could be carried out on the sediment and the boundary layer, which contained high concentrations of catechol amines; in the intermediate layer (layer *C* of Pl. 1, fig. 1), which contained the amines in a large volume of strongly hypertonic sucrose, the method was not applicable, as the sucrose prevented a satisfactory separation by chromatography. In order to avoid this complication, the intermediate layer was re-centrifuged after the sucrose concentration had been reduced by dilution. The chromatographic determination could then be made on the sediment from this centrifugation.

TABLE 4. NAd:Ad ratios and molar ratios amine:ATP before and after centrifugation over 2·0 M sucrose

Expt. no.	Fraction	Ratio, NAd:Ad	Molar ratio, amine:ATP
1	Large granules	0·36	5·7
	Boundary	0·31	5·8
	Sediment	0·75	5·0
2	Large granules	0·24	5·5
	Boundary	0·19	5·4
	Sediment	0·63	5·6

TABLE 5. Distribution of catechol amines and NAd:Ad ratio. Centrifugation over 2·0 M sucrose, followed by re-centrifugation of the intermediate region after adjustment of molarity of sucrose to 1·5 M

Fraction	Catechol amines* (%)	Ratio, NAd:Ad
Large granules	(100)	0·35
A. Uppermost layer of isotonic sucrose	6·2	—
B. Boundary layer	51·4	0·19
C. Intermediate layer	3·9	—
D. Sediment	30·6	0·75
Recovery	92·1	—
C. (1·5 M)†		
Supernatant	1·4	—
Sediment	2·3	0·32
Sum	3·7	

* As percentage of those of large-granule fraction.

† Fraction *C*, after adjustment of molarity of 1·5 M and re-centrifugation.

As shown in Table 5, the total recovery in this experiment was 92 %. To the intermediate layer (fraction *C*) enough 1·0 M sucrose was added to bring the molarity to 1·5 M. Centrifugation in the Spinco ultracentrifuge for 45 min at 142,000 *g* was then carried out.

About one-third of the catechol amine content of the intermediate layer was now found in the supernatant and about two-thirds in the sediment. This showed that the reduction of the molarity of the sucrose solution had resulted in the release of some of the amine from the granules, an effect noted by other observers (Hillarp & Nilson, 1954*b*). The method of Crawford & Outschoorn (1951) was applied to the sediment from this centrifugation. The table shows that the NAd:Ad ratio was intermediate between that of the boundary layer (fraction *B*) and the sediment (fraction *D*) from the first ultracentrifugation.

Distribution of dopamine

The amounts of dopamine present in the homogenates were not sufficient to allow a determination by the bioassay method used for adrenaline and noradrenaline. Homogenates of bovine adrenal medulla were prepared in a saline medium, 9 parts of 0·15 M-NaCl and 1 part of 0·067 M sodium phosphate buffer of pH 7·4. This medium was chosen in preference to the sucrose solution used in all other experiments in order to avoid complications produced by the high sucrose concentration in the chromatographic procedure. The homogenates were centrifuged at 600 *g* for 10 min and the low-speed supernatant thus obtained was used for further analysis. Before the main part of this fraction was centrifuged at high speed, two samples were removed. To one of these samples an equal volume of 1·0 M-HCl was added; part of the sample was then added dropwise to ice-cold 0·01 N-HCl–ethanol. After removal of the precipitated protein by centrifugation, the total catechol amine was determined in the supernatant fluid. This determination gave a control value for recovery of total catechol amines in the second sample, which was treated in the same way as the fractions obtained afterwards. These fractions were: the large-granule fraction (2), the microsome fraction (3) and the non-particulate fraction (1). The large-granule fraction was obtained by centrifugation of the low-speed supernatant for 10 min at 12,000 *g*. The supernatant fluid from this centrifugation was centrifuged for 30 min at 142,000 *g*. The sediment from this centrifugation was the microsome fraction, and the supernatant gave the non-particulate fraction (1).

To each of these fractions 0·5–1·0 ml. of concentrated HCl was added. The protein thus precipitated was removed by centrifugation; the precipitate was washed with 2·0 ml. of water; the washing was added to the supernatant fluid; this was neutralized by adding solid sodium carbonate. The solution was then freeze-dried. Amines were removed by extraction of the residue with 0·5 N-HCl–ethanol. The catechol amine content of each sample was determined according to von Euler & Hamberg (1949).

Ascending chromatography was carried out, using as solvent system *n*-butanol–N-HCl (4:1). After developing the chromatograms for 40–48 hr at 28° C, the dried papers were sprayed with the Prussian blue reagent of Goldenberg, Faber, Alston & Chargaff (1949). This reagent is not specific for catechol amines; it was chosen because of its high sensitivity; it gave a satisfactory reaction with the small amounts of dopamine present in the samples.

On the same sheet, interspersed with the samples, dopamine in 0·01 N-HCl–ethanol was applied to provide a range from 0·25 to 3·0 μg. The amounts of dopamine in the samples were estimated by comparing size and intensity of colour produced by the known amounts of dopamine with those obtained from the samples. The solvent system used was chosen in preference to phenol–HCl, because in the latter dopamine has an R_F value similar to that of ascorbic acid, which also reacts with the Prussian blue reagent. That the spot seen in *n*-butanol–HCl was due to dopamine was confirmed in an experiment in which the material was eluted; the reconcentrated eluate after re-chromatography in phenol–HCl for 20 hr at 20° C gave a spot with the same R_F as a solution of dopamine. This procedure allowed a semi-quantitative determination of dopamine in amounts of from 0·25 to 4·0 μg.

TABLE 6. Distribution of dopamine in relation to that of the total catechol amines. Total catechol amine content in 14 ml. of the untreated low-speed supernatant was 36,400 μg; fractions treated as described in text

	Catechol amines		Dopamine	
Fraction	(μg)	(%)*	(μg)	(%)*
Low-speed supernatant	33,600	—	220	—
(1) Non-particulate fraction	9,720	28·1	70	25·0
(2) Large-granule fraction	24,160	69·8	200	71·4
(3) Microsome fraction	725	2·1	10	3·6
(1) + (2) + (3)	34,605	100·0	280	100·0

* The percentages are given in terms of the total recovery in fractions (1), (2) and (3).

Table 6 gives the results of one of these experiments. It shows that the dopamine represented only a small fraction of the total catechol amines. In five experiments, the mean value of dopamine present was 1·1 % of the total catechol amines. This is a value similar to that found by Shepherd & West (1953) in the bovine adrenal medulla. There was no significant difference in the percentage of dopamine and of the total catechol amine present in the different fractions: about two-thirds was recovered in the large-granule fraction, with most of the remainder in the non-particulate final supernatant; the microsome fraction accounted for only a very small amount of both total amines and dopamine.

In another experiment a large-granule fraction was prepared in isotonic sucrose as described under Methods. One part of the fraction was then re-suspended in 0·3 M sucrose, another part in very hypotonic, 0·1 M, sucrose. The two samples were then recentrifuged. Chromatograms of the sediments and supernatants thus obtained showed that the spot due to dopamine was less conspicuous in the sediment from the hypotonic medium than in the sediment from the isotonic medium; conversely, the dopamine spot had become more conspicuous in the supernatant from the hypotonic medium. In other words, the dopamine had redistributed itself in the hypotonic medium in a way very similar to that known of the other two catechol amines.

DISCUSSION

The results here presented confirm earlier observations that in suspensions of large granules from the bovine adrenal medulla the adrenaline and noradrenaline are similarly divided between supernatant fluid and sediment. On the other hand, in hypertonic sucrose solutions, evidence was obtained of differences in the distribution of the two amines. In each centrifugation of chromaffin granules over 2·0 M sucrose solution, more of the noradrenaline sedimented and was recovered at the bottom of the centrifuge tube and more of the adrenaline was retained above the hypertonic sucrose solution.

Evidence has been obtained by histochemical methods that adrenaline and noradrenaline are differently distributed in the adrenal medulla of many mammalian species (Bänder, 1951; Eränkö, 1955; Hillarp & Hökfelt, 1953). According to these observations, there exists in the chromaffin tissue one type of cell which contains mainly adrenaline; in addition, there exists a second type which contains mainly noradrenaline. The method used in the present work does not make it possible to say whether or not different granules are derived from different cells in the adrenal medulla. However, if the two amines are stored in different cells one might find differences in the granules from the two types of cell. On this view, the imperfect separation of adrenaline and noradrenaline obtained might be explained by assuming that the differences between the granules are too small to allow complete separation. This interpretation has gained in probability through recent work by Schümann (1957), who has followed up the work reported in this paper by a study of the distribution of adrenaline and noradrenaline in homogenates obtained from the adrenal glands of the domestic fowl. Using a specific gravity gradient, he separated a fraction of low specific gravity which was very rich in adrenaline as well as a fraction of granules of high specific gravity which contained mainly noradrenaline.

The method adopted for the study of the distribution of dopamine is less accurate than that used for the study of adrenaline and noradrenaline; dopamine is present in too small amounts and its activity is too low to permit a bioassay on the rat's blood pressure. However, the method used is sufficient to show that the dopamine is distributed between sediment and supernatant fluid in a way similar to the distribution of the other two catechol amines. This is interesting, as it is known that the enzyme dopadecarboxylase, which forms dopamine, is present in the non-particulate fraction (Blaschko *et al.* 1955). The results of this study suggest, therefore, that any amine that arises in the cytoplasmic sap is taken up by the storage granules. It is of interest that Weil-Malherbe & Bone (1957) have recently reported that in brain homogenates the dopamine, which here represents a much larger proportion of the total catechol amines, is distributed very much like adrenaline and noradrenaline.

SUMMARY

1. The distribution of the three catechol amines, adrenaline, noradrenaline and dopamine, in sucrose homogenates of the bovine adrenal medulla has been studied.

2. Centrifugation at high speed of suspensions of large granules in isotonic sucrose over strongly hypertonic sucrose yielded a sediment richer in noradrenaline and poorer in adrenaline than the large granules; conversely, a fraction richer in adrenaline and poorer in noradrenaline was retained in the upper portions of the centrifuge tube.

3. The dopamine present in the gland is distributed over the non-particulate material, the microsomes and the large granules very much as are adrenaline and noradrenaline, with most of the amine in the large granules.

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EXPLANATION OF PLATE

Fig. 1. Appearance of a suspension of large granules of bovine adrenal medulla centrifuged at 142,000 *g* for 1 hr over strongly hypertonic sucrose. The molarity of the hypertonic sucrose solution is given above each tube. The diagram at the right indicates where the tubes were cut after centrifugation: *A*, layer of isotonic sucrose; *B*, boundary layer; *C*, layer of hypertonic sucrose, 'intermediate' layer; *D*, sediment.

Fig. 2. Chromatograms of the boundary layer (fraction *B*) and sediment (fraction *D*), obtained after ultracentrifugation over 2·0 M sucrose of a suspension of large granules of bovine adrenal medulla; the chromatogram of the large-granule fraction is shown on the right. The figure shows, below the origins and above, the spots due to noradrenaline and adrenaline.

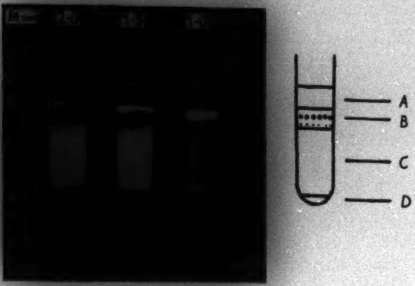

Fig. 1

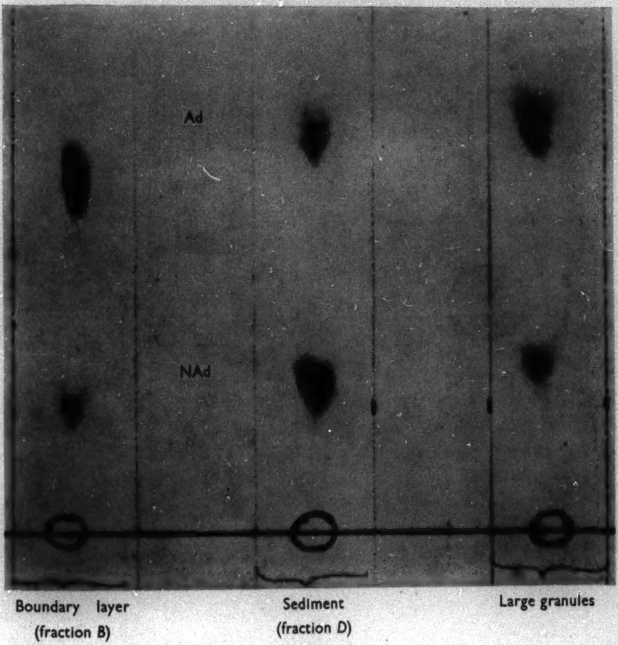
Ad
NAd
Boundary layer (fraction B)
Sediment (fraction D)
Large granules

Fig. 2

(*Facing p.* 192)

PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

PHYSIOLOGY DEPARTMENT, LONDON HOSPITAL MEDICAL COLLEGE, TURNER STREET, LONDON, E. 1

11 *January* 1958

Collection of labelled substances from muscle.

By R. Creese and S. E. E. Hashish. *Department of Physiology, London Hospital Medical College, London, E.* 1

Measurement of reaction times with a conventional scaling unit.

By C. R. B. Joyce. *Department of Pharmacology, London Hospital Medical College, London, E.* 1

Conventional scaling units will measure time very accurately when fed with pulses at constant frequency. The device shown provides such pulses from a multivibrator. The input to the scaler begins when the experimenter exposes a stimulus to the subject, and ceases when the subject responds. The experimenter selects the type of stimulus, the position in which it will be displayed, and the connexions between the stimulus and the response key.

Operation of the circuit (see Fig. 1)

A multivibrator (V_1), supplied from a 150 V stabilized d.c. source, generates a continuous train of voltage impulses at a constant frequency, which can be accurately adjusted by RV_1 to 500 c/sec. The output pulses from one anode (V_{1b}) of the multivibrator are connected as the signal input to a diode gate circuit (V_3) via a cathode follower (V_{2a}). The control input of the diode gate is obtained from the cathode of a thyratron (V_4). The thyratron is normally non-conducting, and the gate is thus kept closed. The experimenter operates P_5 (momentarily connecting the thyratron grid to the cathode via R_{16}). The thyratron conducts, pulses reach the output to the scaling unit through the diode gate and a cathode follower (V_{2b}), until the thyratron is made non-conducting by the operation of the subject's push button (P_{1-4}). This opens the anode circuit of the thyratron, so restoring it to its original state and stopping the pulse train.

When the thyratron conducts, its cathode voltage increases sufficiently to light the selected lamp (L_{1-4}) and L_5 (a visual check for the experimenter). S_2 selects L_4 or an auditory stimulus from a small loudspeaker, *SP*, energized directly from the output pulses.

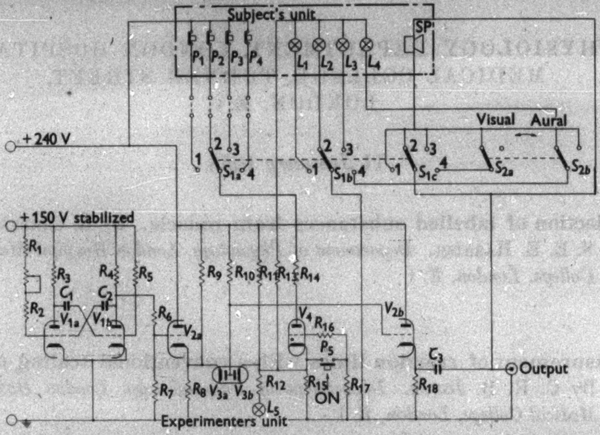
Subject's unit
SP
P1
P2
P3
P4
L1
L2
L3
L4
Visual
Aural
+240 V
S1a
S1b
S1c
S2a
S2b
+150 V stabilized
R1
R2
R3
R4
R5
C1
C2
V1a
V1b
R6
V2a
R9
R10
R11
R13
R14
V4
R16
P5
V2b
C3
Output
R7
R8
V3a
V3b
R12
R15
ON
R17
R18
L5
Experimenters unit

Fig. 1. Circuit diagram. R_1, 22 kΩ, ½ W; R_2, 150 kΩ, 2 W; R_3, 10 kΩ, 1 W; R_4, 10 kΩ, 1 W; R_5, 100 kΩ, 2 W; R_6, 330 kΩ, 1 W; R_7, 1 MΩ, ½ W; R_8, 10 kΩ, ½ W; R_9, 56 kΩ, ½ W; R_{10}, 1 MΩ, ½ W; R_{11}, 82 kΩ, 1 W; R_{12}, 180 kΩ, ¼ W; R_{13}, 120 kΩ, ¼ W; R_{14}, 12 kΩ, 1 W; R_{15}, 12 kΩ, 1 W; R_{16}, 270 kΩ, ¼ W, R_{17}, 270 kΩ, ¼ W, R_{18}, 10 kΩ, ½ W; C_1, 0·02 μF, 350 V; C_2, 0·02 μF, 350 V; C_3, 1 μF, 400 V; RV_1, 15 kΩ, 1 W; V_1, 6J6; V_2, 6SN7; V_3, 6H6; V_4, GT1C; L_{1-5}, are neon indicators type CC7L. +150 V supply neon stabilized using VR 150/30. +240 V supply not stabilized.

The pulse frequency is stable within ± 0·5 %.

I am very grateful to Mr D. A. Austin for designing and constructing the apparatus.

A perfused rat hind-limb preparation. By Jane A. Cutting and J. L. D'Silva. *Department of Physiology, London Hospital Medical College, London, E.* 1

A method of perfusion was required in which the normal water and cation content of the muscles would be maintained. The following procedure gave a preparation which appeared to approach this criterion.

The leg of a rat anaesthetized with sodium amylobarbitone was perfused through the abdominal aorta with heparinized rat blood diluted with Krebs's saline containing 6 % of dextran, to give a haematocrit of 20–25 %. The fluid

TABLE 1

		Water (%)	Sodium (m-equiv/kg)	Potassium (m-equiv/kg)	No. of expts.
Perfused	1 hr	76·4±0·3	21·1±1·5	111·0±5·1	10
	2 hr	76·1±0·45	21·0±1·7	107·1±4·6	7
Control		76·0±0·3	20·3±2·3	110·2±4·3	19

was oxygenated (5% CO_2 and 95% O_2) at 37° C and was perfused at 90–120 mm Hg by means of a pump working at 290 strokes/min. Perfusion was established 5 min after the blood supply had been cut off, and the perfusate was collected through a polythene tube introduced into the inferior vena cava. Other branches of the aorta and iliac arteries were tied.

Table 1 shows sodium, potassium, and water contents of gastrocnemius muscle after perfusion for 1 and 2 hr and for control muscles.

Values of cations are expressed as m-equiv/kg wet muscle. All values are given ±S.D. of individual determinations.

The retention of adaptation to warming and cooling of one hand.

By E. M. GLASER. *Department of Physiology, The London Hospital Medical College, London, E.* 1

The effect of tilting from the horizontal to the tail upwards position on the pressure of the cerebrospinal fluid of the dog.

By T. H. B. BEDFORD. *Department of Pharmacology, University of Manchester*

The effect of tilting from the horizontal position to the tail upwards and tail downwards positions on the pressure of the cerebrospinal fluid has been studied by Weed, Flexner & Clark (1932) and Weed & Flexner (1933*a*, *b*). It would appear from a consideration of their publications that these workers restricted their observations to the immediate effects of tilting on the pressure of the cerebrospinal fluid and that they assumed the new pressure levels were maintained. In the experiments to be described the animals were tilted through an angle of 45° from the horizontal to the tail upwards position and the effect investigated of maintaining them in this position on the pressure of the cerebrospinal fluid. The degree of tilting was restricted to 45°, as it was found that the risk of circulatory failure was thereby reduced. Observations were made on ten dogs.

The animals were anaesthetized with intravenous sodium pentobarbitone and the pressure of the cerebrospinal fluid was recorded in the cisterna magna with an open-end manometer of 1·0 mm bore. The mean arterial blood pressure was measured in the femoral artery. Experiments in which the animals developed signs of circulatory failure were rejected. It was found that the pressure level of the cerebrospinal fluid assumed after tilting is not maintained but begins to fall almost immediately and continues until a level usually slightly higher than the original is reached. This generally occurs after an interval of 15–30 min. If the animal is then returned to the horizontal position, the pressure falls to a level below the original one and then rises slowly towards it. Tilting from the horizontal to the tail upwards position was frequently but not invariably accompanied by a rise of 5–15 mm Hg in the mean arterial blood pressure. The pressure fell rapidly to its original level when the animal was returned to the horizontal position.

Although further work will be necessary before a complete explanation is possible for the fall in cerebrospinal fluid pressure which followed the initial rise after tilting, it is probably caused in part by an increased rate of absorption and this also may account for the low level assumed when the animal is returned to the horizontal position. It would appear from these experiments that the findings of Weed and co-workers are probably only significant when confined to the immediate effect of tilting, as no account was taken of subsequent changes in the pressure of the cerebrospinal fluid.

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Normal levels for plasma adrenaline and noradrenaline compared with those in subjects with complete transverse lesions of the spinal cord.

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In subjects with complete transverse lesions of the spinal cord, the spinal pathways from the higher nervous centres, which are believed to effect control of the sympathetic nerves, are interrupted. It was therefore of interest to determine the adrenaline (Ad) and noradrenaline (NAd) in the blood of such subjects with different levels of lesion, and to follow the change through spinal shock and other conditions of stress.

Fifteen ml. of peripheral venous blood was collected into 5 ml. of a sodium fluoride-thiosulphate solution (Weil-Malherbe & Bone, 1952) and the plasma concentrations of Ad and NAd were determined by a modification of the fluorimetric method used by von Euler & Floding (1955) for urine.

In the past, fluorimetric methods have been criticized for lack of specificity. It was therefore considered necessary to make parallel biological and fluorimetric determinations on peripheral blood. The values obtained for Ad, using the rat uterus, show a good measure of agreement with fluorimetric values. In certain conditions of stress NAd appears to rise to values which can be determined by biological methods and parallel biological and fluorimetric assays in these conditions are in progress.

The plasma Ad and NAd are very low in patients with cervical and high thoracic lesions of long standing (Ad, *ca.* 0·6 μg/l.; NAd, < 0·5 μg/l.). These low values are still present in patients with complete transverse lesions as low as T 3, but below this level there is a marked increase in plasma Ad and NAd. In patients with complete transverse cord lesions below T. 6 the plasma Ad has reached values which do not differ significantly from those found in normal subjects (2·3 μg/l.). The NAd values remain significantly lower than normal in patients with complete cord lesions as low as L 1, but below this level there is a sudden increase to the normal values (4·2 μg/l.).

It is noteworthy that this rise in NAd at L. 3 corresponds to the level of lesions about which paraplegics habitually stand and walk. The sudden assumption of the erect posture is accompanied by a significant increase in plasma NAd in normal subjects but not in paraplegics with complete cervical lesions.

The greatest changes in plasma Ad and NAd were observed in a woman shortly after injury to the cervical cord when increases of 10- to 100-fold above the normal occurred (Ad = 220 μg/l.; NAd = 94 μg/l.). In another case there was no such increase after injury. The reason for this is being investigated.

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Potassium in stimulated muscle.

By R. Creese, S. E. E. Hashish and N. W. Scholes. *Department of Physiology, London Hospital Medical College, London, E. 1, and Department of Pharmacology, Medical Center, University of California at Los Angeles*

Isolated diaphragm muscles can withstand only very low rates of stimulation without a decline in performance. Stimulation at 2/sec produces a marked fall in twitch tension (e.g. Brown, Bülbring & Burns, 1948).

This deterioration is accompanied by a fall in potassium. Diaphragms soaked in saline (Creese, 1954) for 3 hr at 37° C had a potassium content of 93·7 % ± 1·4 (s.e. of 10) when expressed as a fraction of the potassium content of the unsoaked hemidiaphragm, while muscles which were soaked and then stimulated at 2/sec through the nerve for 10–30 min had a potassium content of 79·3 % ± 2·1 (s.e. of 12; $P < 0{\cdot}01$ by *t*-test). Muscles 500–600 μ thick were explored by micro-electrodes which were pushed through the tissue by a motor, and it was found that stimulation at 2/sec for 20–30 min produced depolarization of fibres in the depths of the muscle. These results were similar to the effects produced in resting muscle by reduction in the oxygen content of the gas mixture (Creese, Scholes & Whalen, 1957).

During the first 3 min of stimulation at 2/sec the above signs of deterioration were absent or minimal. Stimulation, however, was accompanied by an increase of 22·6% ± 3·4 (S.E. of 10) in the rate of outward movement of ^{42}K, this being detected in the saline flowing from the muscle. No change was found in the rate of inward movement of ^{42}K during stimulation for 5–10 min, even at 5/sec. The calculation of the net amount of potassium lost per contraction was complicated by the necessity for large and somewhat uncertain corrections which were required to allow for the effects of interfibre diffusion in diaphragm muscle. The estimated value was of the order of 10 pmoles/cm^2/contraction.

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The renal response of conscious dogs to the ingestion of 0·9% sodium chloride. By W. J. O'Connor. *The Department of Physiology, University of Leeds*

The animals used in these experiments were maintained on a diet of meat and biscuits containing about 15 m-equiv of sodium per day, and no additional sodium was given for at least three days before each experiment. In a typical experiment, the rate of excretion of sodium at 10 a.m. was 0·0013 m-equiv/min, urine volume 0·08 ml./min, and urinary sodium concentration 0·017 M. A dose of 250 ml. of 0·9% sodium chloride was given by stomach tube, and 2 hr later sodium excretion was 0·017 m-equiv/min, urine volume 0·12 ml./min and sodium concentration 0·14 M; the increase in sodium excretion was mainly due to increased urinary concentration of sodium rather than to increased volume of the urine. A second dose of 0·9% sodium chloride (350 ml.) was then given and 1½ hr later sodium excretion was 0·28 m-equiv/min, urine volume 1·1 ml./min and sodium concentration 0·25 M; the increase in sodium excretion was now due to increase in the volume of the urine rather than to increase in urinary sodium concentration, as previously described (O'Connor, 1955). The response to ingested saline always presented these different appearances according to the state of saline loading at the time when the dose was given. Administration of saline, with or without saline loading, caused a small increase in the rate of excretion of potassium and a small increase or no obvious change in the excretion of urea.

An attempt has been made to relate the excretion of sodium to changes in the composition of the circulating blood. Ingestion of saline produced no change in the sodium content of the plasma, but caused a fall in the total solid of plasma and the rate of excretion of sodium has been plotted against this measurement. With a fall of plasma solids of up to 0·6 g/100 g below the initial value, sodium excretion increased by about 0·003 m-equiv/min for each fall of 0·1 g/100 g in plasma solids; administration of further doses of saline caused an increase in sodium excretion of 0·07 m-equiv/min for each further fall of 0·1 g/100 g in plasma solids. The first flat part of the curve and the second steep part corresponded to the two different types of response to ingested saline described above.

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An electrical method for recording eye movements. By J. G. Thomas.

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A small probe is used in conjunction with a capacitance gauge of the type described by Alexander (1951). Capacitance changes occur when the eye moves, and these are translated into voltages, which are amplified, and displayed on a cathode-ray tube.

The probe forms one plate of a condenser, and is fixed to a plaster face mask, or to spectacle frames. The other 'plate' of the condenser is (1) the protuberance of the cornea of the open eye, or (2) a contact lens, or (3) a small piece of metal foil stuck to the closed eyelid (in which case, movement of the cornea under the stretched lid causes the foil to tilt).

The sensitivity of the device is equal to that of the best optical methods. Head movements play no disturbing role, so head clamps are not required. Other inherent advantages of an electrical method are ease of setting up, and ease of display and recording. Accurate alinement is not required, and a cathode-ray tube display, with pen recorder, is more convenient to use than a moving photographic film. The one-tube gauge circuit is used with a conventional e.c.g. amplifier, and is duplicated to record movements in two directions.

The device forms the basis of an electrical method for achieving the condition of 'vision with a stabilized retinal image' in which case the outputs of the amplifiers deflect a spot or pattern on a cathode-ray tube, which is being fixated. This has advantages over such optical methods as that of Riggs, Ratliff, Cornsweet & Cornsweet (1953). Other applications and preliminary results will be communicated.

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The work production of man; studies on racing cyclists. By

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Most of the force exerted by a cyclist is used in overcoming air resistance, and since the amount dissipated in mechanical friction is roughly known, the power generated can be estimated if one can measure this aerodynamic drag

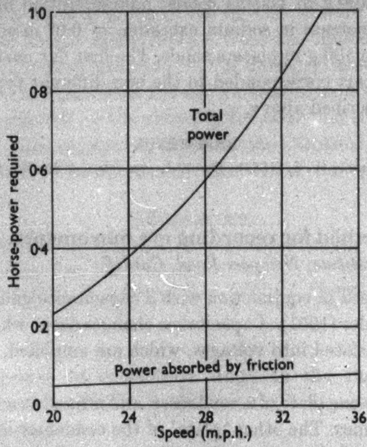

Fig. 1. Power needed to maintain speed in still air on level surface.

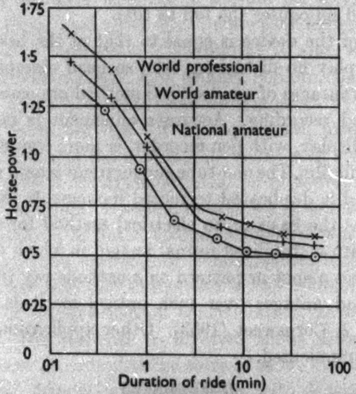

Fig. 2. Power to be generated by an average subject in equalling unpaced cycling records on closed circuits.

Wind tunnel tests have shown that (i) measurements of drag are repeatable within ± 3 %, (ii) the subject's size and his posture in the usual racing position have a relatively slight effect, (iii) drag varies as the square of the speed.

Having thus measured the drag of a subject of average size it is possible to calculate the power he requires to propel himself at various speeds on level ground in still air (Fig. 1). Also, since record speeds over closed circuits of various distances are known, it is possible to suggest with fair accuracy how much power the performers must have generated to achieve such speeds (Fig. 2).

Differences between parts of packed human red cell columns.

By C. R. B. JOYCE. *Department of Pharmacology, London Hospital Medical College, London, E.* 1

Cells were packed by centrifugation from Krebs's saline media containing ^{24}Na or ^{42}K. The concentration of potassium, sodium and ^{24}Na was greatest in the top and least in the bottom third of the columns. The differences in the potassium and sodium concentrations were of the order of 15–25 m-equiv/l. cells and 5–10 m-equiv/l. cells respectively. These observations are not due to chance ($P < 0{\cdot}001$), differences in trapped supernatant volume, or numbers of white cells. Uptake of ^{24}Na and ^{42}K is also greatest over 4 hr by cells in the top third, and of ^{24}Na least in the bottom third. The middle third showed the least exchange of ^{42}K. These observations are also not due to the factors listed above.

Observations on the fat-mobilizing activity of human urine during periods of food deprivation.

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Urine was collected from normal human subjects during the last 24 hr of a 36 hr fast and compared with urine collected from the same subjects during normal feeding. Precipitates obtained from each by the addition of two volumes of ethanol at pH 5·3 were washed repeatedly with ethanol and extracted with 10 ml. of weak sodium carbonate. Precipitation and extraction were then repeated and after neutralization 0·5 ml. of the extracts was injected subcutaneously into mice. The mice injected with fasting urine extracts showed an increase of liver fat at 6–10 hr compared with control animals receiving saline. The non-fasting urine extracts did not increase liver fat.

The fasting extracts were next injected into mice which had incorporated glyceryl tripalmitate-1-^{14}C into their body fat. After 6 hr there was a substantial decrease in the specific activity of the subcutaneous fat as compared with controls injected with saline or non-fasting urine extracts. This effect was attributed to an increased turnover of depot fat.

Two groups of mice were injected with fasting urine extract or with saline every second day for 14 days. Those receiving the fasting urine extract showed a highly significant loss of weight in comparison with the controls, although the food intake was almost identical in the two groups. At the end fat constituted 13·4% of the carcass weight in the controls, but only 11·8% in the group injected with fasting urine extract.

Paper chromatography* of the fasting urine extracts revealed a spot which could not be detected in chromatograms of the non-fasting urine extracts from the same subjects. This material was eluted and found to be highly active in increasing the liver fat in mice.

* By Dr Ivor Smith, Courtauld Institute of Biochemistry.

Carbon monoxide saturation and the recovery of 5-hydroxytryptamine from whole blood. By R. Rodnight. *Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E.* 5

When 5-hydroxytryptamine (5-HT) is added to whole blood less than 40% can normally be recovered by extraction with 19 vol. of acetone, a procedure yielding recoveries of 90–100% when applied to serum or plasma (Hardisty & Stacey, 1955).

In the present work the nature of these losses was investigated. 5-HT was estimated by the rat uterus assay of Amin, Crawford & Gaddum (1954), by the Locarte fluorimeter (Laurence, 1956) or approximately by paper chromatography (Rodnight, in preparation). Low recoveries persisted when 4 vol. of acetone, or in some experiments ethyl alcohol, were used to extract the blood. Inactivation of the 5-HT by enzymes associated with the erythrocytes was excluded by adding it to the acetone used for extraction instead of the blood. In several such experiments recoveries were still in the 20–40% range.

The possibility of losses through oxidation was considered. Blood glutathione and ascorbic acid are both known to be susceptible to oxidation on precipitation of the blood proteins, a reaction which may be prevented by prior saturation of the blood with carbon monoxide (CO) (Numata, 1940; Owen & Iggo, 1956). Applied to the present problem CO saturation raised the observed native 5-HT levels in whole blood by about 60% and enabled from 80–100% of added 5-HT to be recovered. These results were obtained by all three of the methods of estimation referred to above.

The chemical reaction involved in the loss remains obscure; no attempt was made to isolate oxidation products of 5-HT in extracts prepared from blood not saturated with CO. However, from what is known to occur to glutathione and ascorbic acid under similar conditions, it appears probable that oxidation by oxyhaemoglobin is responsible. Apparently this only happens on denaturation of the haemoglobin molecule, as there was no loss when 5-HT was added to haemolysed blood, which was allowed to stand on the bench for 15 min. and then saturated with CO and extracted.

Support for this work from the Research Fund of the Bethlem Royal Hospital and the Maudsley Hospital is gratefully acknowledged.

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Osmotic diuresis during water diuresis.

By Elisabeth Ullmann, *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

In order to reduce antidiuretic hormone activity to a minimum, healthy subjects drank 15 ml. water/kg body weight and maintained the water load for 4–5 hr by drinking as much fluid every 20 min as was excreted in the urine. A period of voluntary overbreathing lasting 80 min was begun 2–2½ hr after the onset of diuresis; alternatively, 250 mg of acetazolamide were ingested at this time.

Both procedures caused a rapid rise in urinary solute output (range of differences: +160 to +890 μosmole/min). Associated with this were: (1) a rise in urine flow (+1·5 to +5·2 ml./min); (2) a rise in urine osmolarity (+3 to +32 m-osmole/l.); (3) a rise in 'free water' excretion (1·1 to +3·7 ml./min).

Welt, Young, Thorup & Burnett (1954), de Wardener & del Greco (1955), and Kleeman, Epstein & White (1956) have previously reported the same combination of changes, but for conditions in which disease or changes in adreno-cortical activity, G.F.R., or plasma osmolarity might have modified tubular function.

The present experiments show that none of these factors need be implicated and that the observed paradoxical excretory pattern can develop in normal subjects, without pre-treatment, during a physiological rise in urinary solute output which is the result of diminished tubular reabsorption of filtered electrolyte.

It seems probable that the simultaneous rise in 'free water' excretion and urine osmolarity is directly connected with an increased inflow of isotonic fluid from the upper nephron into the diluting segment (Wirz, 1956).

Increased 'free water' excretion in these circumstances could be the result of increased reabsorption of solute without water; but it is difficult to reconcile this hypothesis with the excretion of additional 'free water' during forced breathing and after acetazolamide.

Instead, it is suggested that reabsorption of solute without water may proceed at constant rate in the diluting segment, irrespective of the volume of isotonic fluid entering it. A constant volume of 'free water' would thus be formed of which more and more would escape subsequent reabsorption as the inflow of isotonic fluid into the segment, and hence the total rate of flow through it, increases. This would result in increased 'free water' excretion coupled with a rise in urinary osmolarity.

This hypothesis implies, however, that at least as much 'free water' as the largest increments observed, i.e. 4–8 ml./min—and perhaps more—can be reabsorbed from a hypotonic tubular fluid even when ADH activity is minimal.

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Costocervical and thyrocervical contributions to the blood supply of the cat brain. By R. L. Holmes and J. H. Wolstencroft. *From the Departments of Anatomy and Physiology, University of Leeds*

Linzell & Waites (1957) have shown that medullary function in sheep and goats continued after tying the common carotid and vertebral arteries on both sides and they explained their results in terms of anastomoses between spinal and vertebral arteries. While making similar experiments in cats we investigated the contribution of the costocervical and thyrocervical trunks to medullary blood supply.

Respiration and blood pressure were recorded in cats anaesthetized with sodium pentobarbital (45 mg/kg). Snares were placed around the common carotid and vertebral arteries and also the thyrocervical and costocervical trunks of both sides, close to their origin from the subclavian, before occlusion by clips.

In six of nine animals respiration was maintained (although usually reduced) after occlusion of both carotid and both vertebral arteries, but was abolished (with one exception) by the subsequent occlusion of thyrocervical and costocervical trunks on both sides. In the other three animals respiration ceased after occlusion of both carotid and both vertebral vessels.

In four animals of the series the arteries were injected with latex (60 %) after the experiment and dissections were made following fixation.

Considerable variation in the pattern of arterial branches was found. In each of the four animals one or more arterial branches from the thyrocervical trunk passed medially along the upper nerves forming the brachial plexus and apparently entered the spinal canal. Small branches of costocervical origin were similarly related to the lower nerves of the plexus and a larger branch usually ascended close to the vertebral artery.

It was not possible in this small series to correlate any major variation in the pattern of the dissectable arteries with the adequacy or inadequacy of the medullary blood supply following occlusion of the main vessels. It is possible that this depends on anastomoses between the spinal vessels and minute branches passing medially along the nerve roots; or alternatively on anastomoses between vertebral and other subclavian branches within the dorsal musculature.

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The liberation of ATP from perfused rabbits' ears on antidromic stimulation of the sensory nerve.

By PAMELA HOLTON. *Department of Physiology, St Mary's Hospital Medical School, London, W.* 2

It was previously reported (Holton & Holton, 1953) that the optical density of the perfusate from the isolated rabbit's ear increased specifically at 260 mμ when the great auricular nerve was stimulated antidromically. These results, together with other evidence (Holton & Holton, 1954) suggested that adenosine triphosphate (ATP) might be liberated when sensory nerve fibres were stimulated antidromically. The evidence in favour of this hypothesis has now been strengthened by measuring ATP using the specific fire-fly luminescence method.

During control periods the perfusate from rabbits' ears contained no detectable ATP (<2 pmole/ml.) but during and immediately after stimulation the ATP concentration rose (2·5–80 pmole/ml.). The amounts of ATP appearing in the venous outflow were smaller than would be expected if the optical density increase were due to ATP alone. In order to see what proportion of ATP disappeared during perfusion through the tissues, known amounts were injected into the arterial cannula. It was found that 0·5–16% (depending on the perfusion rate) could be recovered from the venous outflow as ATP, but that about 60% was recovered as nucleotide as measured by optical density. These results show that most of the injected ATP was broken down, and it follows that the ATP which reaches the venous outflow after stimulation probably represents only a small fraction of what is liberated in the tissues.

The source of the ATP has not yet been identified. The possibility that it comes from haemolysed blood has been examined and shown to be unlikely.

These results are in favour of the hypothesis that ATP is the substance responsible for antidromic vasodilation.

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Effects of 5-hydroxytryptamine and 5-hydroxytryptophane on defaecation and propulsion of a charcoal meal in intact mice.

By R. T. Brittain and H. O. J. Collier. *Research Division, Allen and Hanburys Ltd., Ware, Herts*

5-hydroxytryptamine (5-HT) and 5-hydroxytryptophane (5-HTP) stimulate peristalsis in isolated guinea-pig's ileum (Bülbring & Lin, 1957). Both substances have been briefly reported to promote defaecation (Erspamer, 1952; Lembeck, 1957; Collier, 1957; Woolley, van Winkle & Shaw, 1957). Since several investigators have suggested that 5-HT may participate in natural propulsion of gut contents, the effects of 5-HT and 5-HTP on this function have been further studied in intact mice.

Table 1. Defaecation and propulsion of a charcoal meal in intact mice after subcutaneous treatment with 5-HT or 5-HTP

Treatment (/kg)	Mean values ±s.e. obtained for periods after treatment						
	Weight (mg) of faeces passed			Percentage small intestine traversed			
	0–30 min	30–60 min	90–120 min	0–20 min	30–50 min	60–80 min	120–140 min
Saline 20 ml.	12·5±6·8	17·5±8·6	17·5±8·6	59·3±6·1	70·9±11·2	74·4±3·6	58·5±5·7
5-HT 10 mg	208·8±22·7	6·3±1·8	0	34·7±4·4	33·6±3·3	44·0±3·2	55·0±5·5
dl-5-HTP 100 mg	231·3±27·2	16·3±8·4	0	60·7±2·8	58·9±5·8	68·2±4·5	50·2±4·6

In all experiments, mice were treated subcutaneously with 5-HT, dl-5-HTP or 0·9% NaCl in water. In experiments on defaecation, immediately after treatment mice were placed in individual wire mesh cages without food or water and faeces were collected, counted and weighed at consecutive 30 min intervals. Both 5-HT and 5-HTP first increased and then reduced the number and weight of faeces produced (Table 1). 10 mg/kg 5-HT or 100 mg/kg 5-HTP were highly effective, 1 mg/kg 5-HT or 10 mg/kg 5-HTP just effective and 0·1 mg/kg 5-HT or 1 mg/kg 5-HTP ineffective. The relationship of log dose 5-HT or 5-HTP to mean weight of faeces passed within 30 min could provide a basis for studying mimetics or antagonists of 5-HT.

In further experiments, at various times after treatment, each mouse received orally 0·4 ml. 5% suspension of charcoal in 10% gum acacia in water. 20 min later, mice were killed and the distance the charcoal had travelled from the pyloric sphincter was compared with the total length of the small intestine. Up to 100 mg/kg 5-HTP failed to influence significantly the distance travelled (Table 1); but 5-HT (10 mg/kg) reduced this distance. This retardation probably occurred at the pyloric sphincter, since the effect disappeared if charcoal was allowed to enter the duodenum before 5-HT was given. The difference in action between 5-HT and 5-HTP was unexpected.

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OBSERVATIONS ON THE NATURE OF THE CHROMAFFIN REACTION

By A. J. HALE*

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In the chromaffin reaction a brown colour is produced in fresh tissue by the action of fixatives containing bichromates (Henle, 1865). In the adrenal medulla the reaction is considered to be produced by adrenaline (Hultgren & Andersson, 1899).

Borberg (1913) thought that the reaction was caused by the reduction of bichromate to chrome yellow by adrenaline and Ogata & Ogata (1923) have attributed to this reduction product the formula $(CrO_2)_n$.

Verne (1923) pointed out that not only adrenaline but all those substances which contained polyphenols, aminophenols or polyamines with the hydroxyl group in ortho or para positions would give the reaction. Gerard, Cordier & Lison (1930) have suggested that the reaction is due to the appearance of an oxidation product of the reacting substance rather than a reduction product of the bichromate. They support this argument with evidence that a brownish colour, similar to that which is produced by bichromate, can be produced by the action of iodates. They suggest that the brown colour is caused by the oxidation of the reacting substance to a quinone or quinoneimine and the combination of such an oxidation product with one of the unaltered polyphenols of the reacting substance, to give a quinhydrone. Verne (1923) points out that a chromaffin reaction, produced by oxidizing agents other than bichromate, has a less stable colour complex which is not identical to that produced by bichromate.

Since it is possible that a precipitate of chromium dioxide might be produced during the oxidation reaction (Gerard *et al.* 1930; Lison, 1953), it is possible that chromium might be deposited in the tissues irrespective of the mode of formation or the coloured substance. Coupland (1954) has, however, shown that chromium is deposited in the adrenal medulla and that it remains there even when the colour of the chromaffin reaction is removed by exposure

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to bromine water. But he does not show the localization of the chromium within the medulla.

Chromium has a mass absorption coefficient for soft and ultra-soft X-rays which is approximately double that of the proteins, lipids and other components of a normal cell (Text-fig. 1). Thus, if chromium is present in a tissue, one would expect to get a heavy absorption of soft X-rays there. Utilizing the X-ray micro-technique of Engström (1946) and Engström & Lindström (1950), I have therefore examined tissues stained by the chromaffin reaction to see if chromium is present in them and if so, where it is located.

METHODS

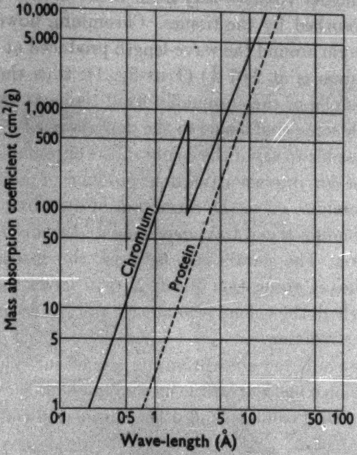
Adrenals were removed from rats which had been killed by a sharp blow on the head. Some were fixed in 10% neutral formalin (24 hr) and some in 3·5% potassium bichromate (24 hr) followed by 10% neutral formalin (24 hr). After fixation the tissues were washed (24 hr in running water), dehydrated, cleared, embedded and blocked in paraffin wax. The tissues were arranged so that each block contained at least one adrenal fixed by each method. Sections 4 and 20 μ in thickness were cut and mounted on Kodak maximum resolution plate in the dark room, according to the technique of Greulich & Engström (1956). Some sections were also mounted and counterstained by conventional methods.

The plates, with sections, were exposed in a miniature X-ray tube fitted with an aluminium window (filter) 1000 Å thick (Engström & Lundberg, 1957). The sections 4 μ in thickness were exposed for 40 min at 1·0 kV and 1·0 mA and for 3 hr at 0·8 kV and 1·0 mA. The sections 20 μ in thickness were exposed for 40 min at 1·5 kV and 1·0 mA. These voltages in this tube provide polychromatic X-rays which have a short wave-length limit of about 12 Å (1·0 kV), 15 Å (0·8 kV) and 8 Å (1·5 kV). Some of the sections 20 μ in thickness were also exposed in a sealed-off X-ray tube (Machlett OEG–50) for 8 min at 7–8 kV and 8 mA. This provides filtered X-rays having a maximum intensity around the wave-length 2·0 Å but with a short wave-length limit of about 1·5 Å. This wave-length is hardly absorbed by protoplasm but is considerably absorbed by chromium. After exposure the sections were removed by immersing the plate in acetone and the plates were developed in Kodak 19B developer for 5 min.

The relative amounts of absorption produced by the images of the different tissues on the X-ray were measured in a microdensitometer.

RESULTS

In adrenals fixed in bichromate there is a strong chromaffin reaction in all the cells of the medulla (Pl. 1, figs. 1, 2) except in ganglion cells seen in some sections. This chromaffin tissue shows a very heavy absorption of X-rays at all the wave-lengths at which it is examined (Pl. 1, fig. 3). This high absorption is markedly different from the very low absorption of the medullary cells seen in the tissue fixed in neutral formalin (Pl. 1, fig. 4). Examination of the heavily absorbing chromaffin cells at high magnification shows that the increased X-ray absorption is marked in the nuclei and also in the cytoplasm of many of the medullary cells (Pl. 2, fig. 1). This distribution of highly absorbing areas corresponds to that of the chromaffin reaction. The ganglion cells show a very low absorption similar to that seen in ganglion cells fixed in formalin (Pl. 2, fig. 2).



Text-fig. 1. The mass absorption coefficient of chromium and protein as a function of wave-length; log.–log. scale.

DISCUSSION

Coupland (1954), using autoradiography of tissue fixed in potassium bichromate prepared from radioactive chromium, has shown that the chromaffin reaction is associated with the deposition of chromium in the medulla. He could not, however, localize the deposition of the chromium within the medulla because of the lack of resolution in the autoradiographic technique. Using the diphenylcarbazide method he measured the amount of chromium present (per dry weight: personal communication) in isolated medullary and cortical tissues and found that there was approximately four times as much chromium present in the medulla as in the cortex after conventional histological treatment.

Lindström (1955) found that the medullary to cortical ratio of relative X-ray absorptions in frozen-dried rat adrenal was 1:4. In the present investigation the ratio is approximately 2:3 for tissues fixed in formalin. In adrenals fixed in bichromate the ratio is altered to approximately 4:3. This marked increase in the ratio of the absorptions indicates that a large amount of chromium is deposited in the medullary tissue. The increase in absorption occurs in the nucleus and the cytoplasm and is marked in those edges of the cells which border on the venous sinuses.

I have used relatively high voltages for some sections since, at lower voltages, absorption might have been due to some cause other than the deposition of chromium, e.g. cell shrinkage and thus increase in dry mass per unit volume. At higher voltages very little of the X-rays which have a short wave-length is absorbed by the tissues. Chromium, however, has a marked absorption maximum around the wave-length produced at the higher voltages (*K*-edge of chromium is at 2·07 Å) (Text-fig. 1): thus the heavy absorption pattern in the medulla of the chromaffin-fixed tissue at this wave-length can be attributed with some confidence to the deposition of chromium.

It is still not possible to say if the colour of the chromaffin reaction is due to the production of an organic oxidation product of adrenaline, or to the deposition of chromium, or to the formation of an adrenochrome-chromium complex: but chromium is certainly deposited and firmly bound at the site of the colour reaction. The parallelism between the intensity of the colour reaction and the heavy absorption of soft X-rays shows that the chromium is actually present where the colour is produced and is not just deposited in the medulla during the reaction.

Since the increase in X-ray absorption can be measured accurately, it might be possible to develop a method for the measurement of the amount of reacting material in individual medullary and other chromaffin cells.

SUMMARY

1. Sections of adrenals fixed to demonstrate the chromaffin reaction were examined by ultra-soft X-ray methods to determine if chromium was deposited at the site of the reaction.

2. It was found that chromium was deposited in the cytoplasm and nuclei of the medullary cells.

I should like to thank Professor Arne Engström of the Department of Medical Physics, Karolinska Institutet, Stockholm, for letting me use his laboratory facilities, and the Wellcome Trust for a travel and maintenance grant which allowed me to carry out the investigation.

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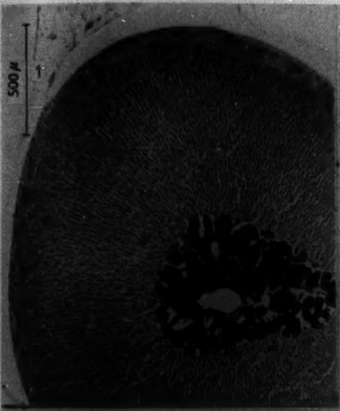
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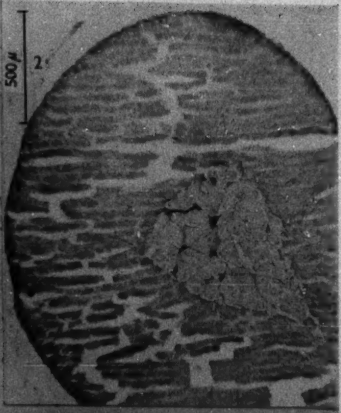
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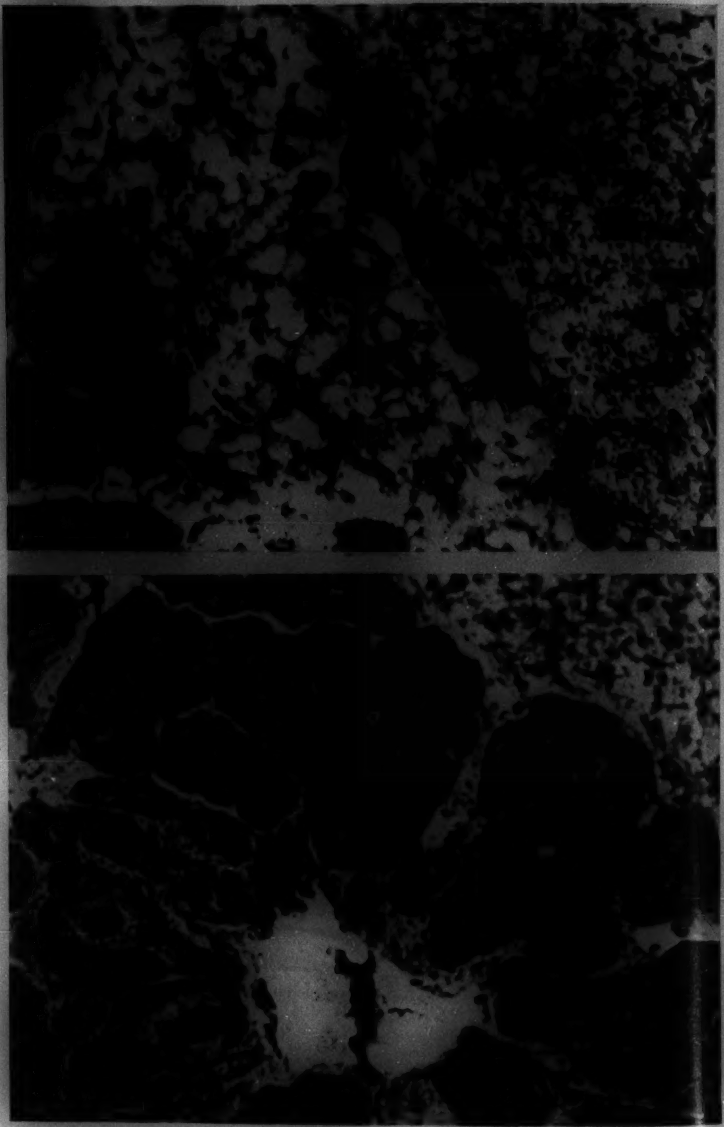

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Section 20 μ in thickness of adrenal fixed in 3·5% potassium bichromate. Counter-stained with haematoxylin.

Fig. 2. Section 20 μ in thickness of adrenal fixed in 10% neutral formalin. Stained with haematoxylin.

Fig. 3. Microradiograph of 4 μ section of adrenal fixed in 3·5% potassium bichromate. Exposed for 3 hr at 0·8 kV and 1·0 mA. Areas of high absorption are light.

Fig. 4. Microradiography of 4 μ section of adrenal fixed in 10% neutral formalin. Exposed for 3 hr at 0·8 kV and 1·0 mA. Areas of high absorption are light. These semi-serial sections were mounted under dark-room conditions where it was difficult to avoid overspreading or folding of the tissue.

PLATE 2

Fig. 1. This is the preparation, shown in Pl. 1, fig. 3, at a higher magnification. The large dark areas to the left are ganglion cells. Light areas are heavily absorbing.

Fig. 2. This is the preparation, shown in Pl. 1, fig. 4, at a higher magnification. The light mass in the centre consists of densely packed blood corpuscles and plasma. The large cells to the left of it are ganglion cells. Cortical tissue lies to the right. Light areas are heavily absorbing.

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A STUDY OF RAPID MECHANICAL EVENTS IN A MECHANORECEPTOR

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All sensory receptors have a functional similarity in causing action potentials to arise in their afferent nerves in spite of the diversity of specific stimuli. An electrical precursor of these action potentials—the receptor potential—has been described for receptors of many modalities and, in particular, for amphibian (Katz, 1950), mammalian (Gray & Sato, 1953) and crustacean (Eyzaguirre & Kuffler, 1955) mechanoreceptors. In all these cases the mechanical quantities given were those describing the specific stimulus, the amount of stretch or its rate of application, for example; the stimulus, however, is by definition an event external to the receptor and the mechanical energy associated with it undergoes transformation on its way through the receptor to the sensitive elements from which the receptor potential arises. The nature of this transformation and, hence, the way in which the stimulus is modified before it reaches the sensitive elements depends directly on the mechanical properties of the particular receptor; it is, therefore, an essential link in the chain of events connecting stimulus and response. The purpose of this investigation was to display the effect of this transformation by describing the internal mechanical changes in a mechanoreceptor due to external mechanical changes which might reasonably be related to the specific stimulus for that receptor. The theoretical advantage of such an investigation is twofold; on the input (stimulus) side it might provide an explanation for general receptor properties such as adaptation and specific thresholds, while on the output (receptor potential) side it might give more information about the physical mechanisms previously suggested for producing the receptor potential (Katz, 1950; Gray & Sato, 1953).

The choice of receptor for this sort of investigation must be guided by the availability of evidence for a well-defined receptor potential and by the techniques available for identifying changes other than electrical in the receptor. The mesenteric Pacinian corpuscle satisfies both of these requirements since there is a great deal of evidence relating to the first (Gray & Sato, 1953), and the second requires only conventional methods of photomicrography to observe displacements within the corpuscle resulting from externally applied compression. The experimental situation is made easier, too, by the regular and laminated structure of the corpuscle through the greater part of its volume so that the preparation might be thought of as having its own 'built-in' measuring scale.

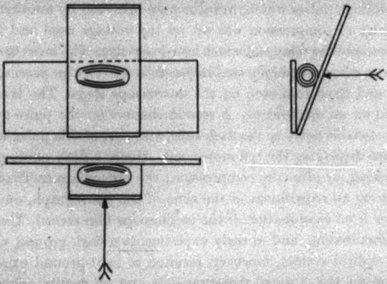
The work described in this paper deals with the structure of the corpuscle at rest and when subjected to one particular kind of deformation; a preliminary account of it has already been given (Hubbard, 1957).

METHODS

The methods used allowed two aspects of the internal structure of the corpuscle to be considered. The first, and most important, was the measurement of the displacements of individual lamellae during a rapid compression applied externally; however, from the same records the spacing between successive lamellae could also be obtained.

Preparations, apparatus and experimental arrangements

The compression used for studying the mechanical response of the corpuscle was a linearly rising one and the method was to take brief (2–5 μsec) flash photomicrographs of the corpuscle during and after the compression. The corpuscle was dissected free from the mesentery of the cat and cleared of surrounding fat particles, and the afferent nerve cut as near to the corpuscle as possible so as not to obscure subsequent photographs. A glass cell (Text-fig. 1) in the form of a hinge was made of microscope slides and sealed with latex-backed cellophane ('Sellotape' was found to be the most effective). The cell was about three-quarters filled with Ringer-Locke's solution (NaCl 0·9, KCl 0·042, $CaCl_2$ 0·024, $NaHCO_3$ 0·015 %, w/v) at room temperature and the corpuscle placed in it, being allowed to sink until it assumed the position shown in the figure. Its orientation was usually as shown in the figure but sometimes the long axis was set vertically. (No significant difference was observed in the results for either position.)



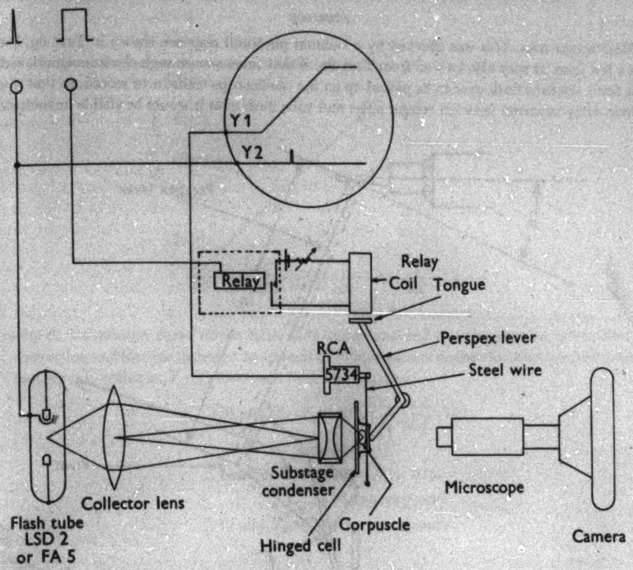
Text-fig. 1. Diagram of the glass cell in which the corpuscle was compressed. (Sealing material not shown for clarity.) The corpuscle is shown in the most usual orientation. The arrow indicates the coincidence of the line of compression and the optical axis.

This particular arrangement will be considered again in the Discussion. Here it is only necessary to recall that a most noticeable and constant feature of the corpuscle in its mesenteric environment is that it lies with its long axis in the plane of the mesentery with connective tissue sheets passing over it on both sides. It is clear that a displacement of these sheets will submit the outer capsule of the corpuscle to deformations similar to those which are produced by the cell shown in the figure. The near coincidence of the planes of compression and observation is justified both by the nature of the problem and by considerations of technique. In the initial stages of the work it was essential that the plane of observation contained the long axis of the corpuscle, since a question of primary significance was the possibility of longitudinal extension of the single nerve fibre within the corpuscle. Subsequent results and the desirability of the best possible resolution near the core offered a strong argument to keep to the original arrangement; if the long axis were viewed end-on, for example, resolution would be worse since the specimen would be nearly twice as thick, with a consequent increase in optical density on either side of the plane of observation. The only other possibility is a rotation of this plane about the long axis of the corpuscle and this would be technically more difficult than the existing arrangement. In fact, it would not be likely to give results differing greatly from those obtained, since the corpuscle is remarkably symmetrical about its long axis and since its contents are, largely, fluid and incompressible; thus, an extension in one plane would appear as a shortening in the plane at right angles. The cell was placed on the stage of a microscope mounted horizontally on an optical bench and a camera and adapter fitted in place of the microscope eyepiece. The average size of corpuscle used in these experiments was about 1200 μ long and 800 μ in diameter.

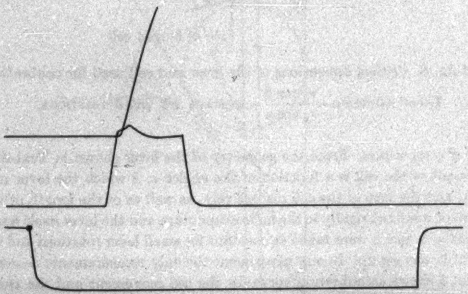
The light source was an Arditron flash tube with its associated equipment giving a high-intensity flash of about 2 μsec duration. The tube was later changed to a BTH FA5 which gave rather longer flash times (about 5μ sec) but had the advantage that its repetition rate was determined only by the charging circuit of the power pack. During most experiments photographs were taken at about 20 sec intervals.

The arrangement of the optical and electrical apparatus is shown in Text-fig. 2. The cell movement, and hence the compression applied to the corpuscle, was controlled by a Perspex lever having a relay tongue fixed to one end. The relay coil was mounted adjustably above this and was driven by sharp voltage pulses which, in turn, were operated by another relay working off a pulse generator. Velocity of compression was set by the voltage used and the total amount of movement by the position of the relay coil which acted as a stop. The movement was measured by an RCA 5734 transducer whose anode pin was fastened to a steel wire passing behind the moving front half of the cell and fixed to a stop on the microscope stage. The transducer output was amplified and recorded on an oscilloscope. A second channel on the pulse generator provided a trigger pulse having a variable delay to the flash tube, and part of this pulse was fed to the second beam of the oscilloscope displaying the cell movement. Hence a flash photograph could be taken at any time before, during, or after the compression; the immediate oscilloscope display gave a rough visual guide during an experiment of the time of the photograph, and accurate measurement was possible later from examination of the oscilloscope film record. The optical system was adjusted for Köhler illumination, and in early experiments a dark-ground wheel stop was used. Improvements in the optical system, however, resulted in light-ground exposures giving better contrast and resolution for the required measurements and all results quoted in this paper are from such exposures.

Calibration. At the end of each experiment a time trace was added to one beam of the oscilloscope and photographed. Calibration for vertical deflexion, that is, for transducer output and hence for corpuscle compression, was obtained by advancing a micrometer screw head on to the lever in several stages, taking an oscilloscope record at each stage. These records gave a check on the linearity of the transducer and also gave, together with the time trace, the means of estimating the maximum size and mean velocity of the compression. At each stage also a photomicrograph of the corpuscle was taken and measurements of these were used to calculate the 'equivalent static displacement' described below. Absolute magnification was measured by photographing a micrometer slide mounted in an equivalent position to the cell; since the microscope draw tube length was never altered the film so obtained was simply measured at the same time as the corpuscle negatives were measured.


Y1
Y2
Relay
Relay
Coil
Tongue
Perspex lever
RCA
5734
Steel wire
Substage condenser
Microscope
Collector lens
Flash tube
LSD 2
or FA 5
Corpuscle
Camera
Hinged cell

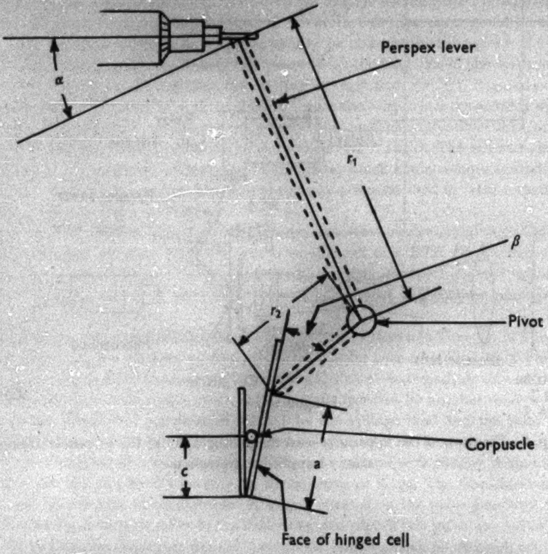
Text-fig. 2. Diagram of the apparatus used for photography of the corpuscle during and after an applied compression.



Text-fig. 3. The delay between flash trigger pulse and tube flash-over (redrawn from photograph). Top trace, vacuum photocell response; middle trace, flash trigger pulse; bottom trace, time pulse of 1 msec.

Accuracy

Flash trigger time. This was checked by a vacuum photocell response shown in Text-fig. 3 and was a few μsec. It may also be seen from Text-fig. 8 that there was enough electromagnetic radiation from the tube flash-over to be picked up on the oscilloscope transducer record, so that if any serious delay occurred between trigger pulse and tube flash-over it would be visible immediately.

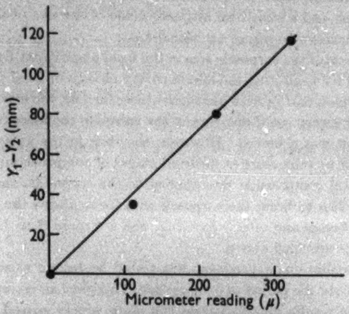


Text-fig. 4. Critical dimensions of the lever and cell used for calibration.

$$\text{Lever reduction} = \frac{r_2 \cos\beta}{r_1 \cos\alpha} = \text{constant for small rotations.}$$

Total amount of compression. From the geometry of the lever shown in Text-fig. 4 it may be seen that movement of the cell is a function of the angles α, β which the lever makes with the micrometer head and the face of the cell respectively, as well as of the length of the arms r_1, r_2. Since the lever pivot was fixed rigidly to the microscope stage and the lever itself was always in the same mean position, α and β were taken as constant for small lever rotations and were measured when the apparatus was set up. In any experiment the only measurements necessary were thus a and c. Text-fig. 5 shows a calibration curve for the cell movement and the steps involved in calculating the compression directly in μ. Occasionally, calibration was checked by using a micrometer head directly on to the face of the cell, approaching obliquely so as to reduce the forward advance of 10μ per micrometer division to give a reasonable number of divisions to correspond to the total compression.

Errors in measured displacements. Displacements of the lamellae measured from photomicrographs were likely to have the largest errors in (a) the process of measuring the projected negatives, and (b) optical factors due to change of focal plane with compression.



Text-fig. 5. Calibration curve of the RCA 5734 transducer and a representative calculation for converting oscilloscope deflexion to amount of compression of corpuscle. Abscissa, micrometer reading (μ); ordinate, Y_1-Y_2 measured from film (mm).

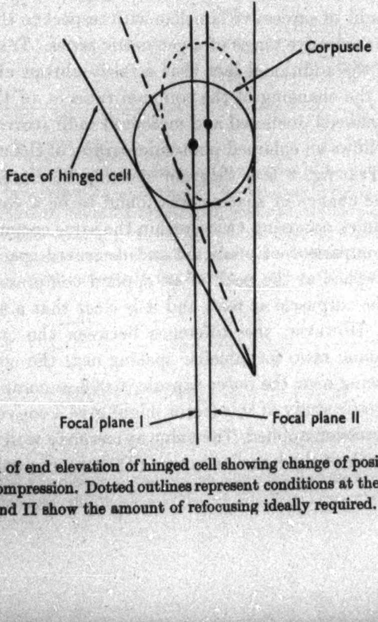
$$120 \text{ mm } Y_1-Y_2 \equiv 320\,\mu;\ \frac{r_1 \cos\alpha}{r_2 \cos\beta}=5/2;$$

$$a/c=16/7$$

$$\therefore 120 \text{ mm} \equiv 320\times 2/5\times 7/16.$$

$$=56\,\mu \text{ at corpuscle,}$$

$$\therefore 10 \text{ mm } Y_1-Y_2 \equiv 4{\cdot}7\,\mu \text{ at corpuscle.}$$



Text-fig. 6. Diagram of end elevation of hinged cell showing change of position of the axis of the corpuscle with compression. Dotted outlines represent conditions at the peak of compression. Focal planes I and II show the amount of refocusing ideally required.

(*a*) The accuracy of measurement was examined by repeated random readings of one lamella in one projected photograph and a statistical estimate made of the size of the error. The results of this procedure gave a mean value whose S.E. was 0·4 μ.

(*b*) Ideally, the long axis of the corpuscle was in the focal plane (Text-fig. 6), and it is clear that conditions were optimal for neglecting the effects of any change of focal plane since the lamellae cut the plane at right angles, that is, with least curvature; further, the intensity of the light source used was such that only a very small aperture in the substage condenser was required, so that a maximum depth of focus was achieved. However, the shift in the axis of the corpuscle under compression was checked by refocusing at different stages of compression; in one experiment, for example, where 60 μ total compression was applied to the corpuscle, the microscope objective required an advance of 24 μ to bring the corpuscle into focus again; the difference in measured displacement, however, before and after refocusing, was not more than 1 μ, that is, 2 S.E. of the accuracy of measurement specified above.

Finally, consistency within one experiment was tested by taking closely spaced flash photographs of one corpuscle over the period of compression. This gave an estimate of the limits which could be assigned to the results obtained from the more widely spaced photographs taken in the average experiment. It was found that all displacements measured lay within ± 1·5 μ of an estimated mean curve.

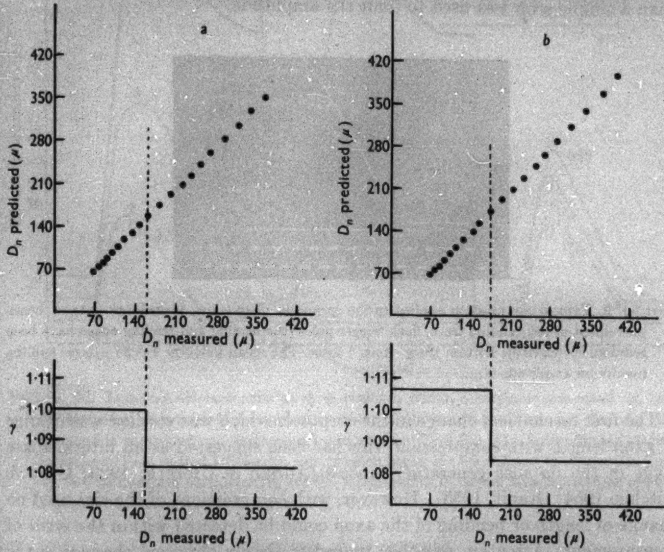
RESULTS

Spacing of the lamellae

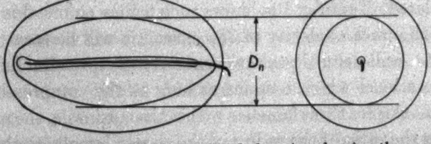
The non-uniform spacing of the lamellae was noted by Gray & Sato (1955) and used by them to explain the discrepancy between theory and experiment in studies on the diffusion of ions through the corpuscle. Measurement of lamellar spacing from photomicrographs of several corpuscles taken during the experiments described above showed that the spacing was logarithmic, that is, that the radii of successive lamellae with respect to the long axis could be defined by the successive terms of a geometric series. Text-fig. 7*a* shows a typical case with the additional fact that a fairly abrupt change of spacing necessitated only the changing of the common ratio, γ, of the series to keep good agreement between predicted and measured radii (correlation coefficient 0·9996). Plate 1 shows an enlarged photomicrograph of the corpuscle used for this figure, and Text-fig. 8 is a diagram showing how measurements were made from it. The change of spacing was found to be a common feature of corpuscles, sometimes occurring twice within the same corpuscle. Text-fig. 7*b* shows the same comparison of predicted and measured spacing for the same corpuscle photographed at the peak of an applied compression (Text-fig. 7*a* was taken with the corpuscle at rest) and it is clear that a similar change of spacing remains. However, the difference between the 'rest' and 'peak' values of the common ratio for lamellar spacing near the core is more than twice that for spacing near the outer capsule (0·008 as compared with 0·003) and a more systematic study of this factor might give a convenient parameter to define the compression applied. The value at rest may well be related to the way in which growth of the lamellae occurs (Thompson, 1942).

The response to compression

The time course of a typical compression is shown in Text-fig. 9, together with a time trace and the flash trigger pulse. As stated in the Introduction, the compression used was one which 'might reasonably be related to the specific stimulus' for the corpuscle; hence, values of 19–90 μ maximum amplitude and of 2·5–40 μ/msec mean velocity have been used. These would correspond to a



Text-fig. 7. Comparison between measured and predicted spacing of the lamellae using the formula $\ln D_n = \ln D_o + n \ln \gamma$, where D_n = diameter of the nth lamella and D_o and γ are constants. *a*, top graph: abscissa, D_n measured; ordinate, D_n predicted; bottom graph: abscissa, D_n measured; ordinate, mean value of γ calculated from the formula. Corpuscle at rest. *b*, axes as in *a*, the same corpuscle at peak of applied pulse of mean velocity 19 μ/msec and maximum amplitude 60 μ. Dotted ordinate is the lamella at which an abrupt change of spacing occurred.

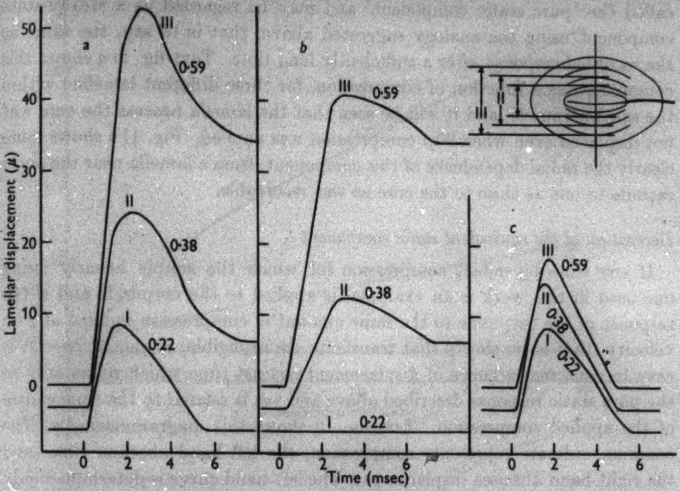


Text-fig. 8. Diagram of a corpuscle in side and end elevation showing the measurements made. D_n represents the diameter of the nth lamella out from the core.

stimulus at least 40 times threshold and 2·5 times the critical slope respectively (Gray & Matthews, 1951). The figure shows that at high mean velocities ringing occurred in the mechanical system, but when this occurred the amplitude of the oscillation was always kept to a small fraction of the total. The tube flash-over may be seen, as mentioned earlier. It will be noted that the rising front of the compression takes off more sharply than the levelling out at the peak; since this paper deals only with 'on' effects no special device other than a simple stop was used to limit the amplitude.

Text-fig. 9. Typical compression applied to the corpuscle. Top trace, transducer output, hence movement of cell; middle trace, flash trigger pulse (the leading and trailing edges have been redrawn for clarity); bottom trace, time, 1 msec. The mean velocity was 35 μ/msec and the maximum amplitude 90 μ.

The first mechanical change in the corpuscle which was studied was change of axon length with compression; this has been suggested as an intermediate stage in the impulse generating process (Adrian & Umrath, 1929; Gray & Ritchie, 1954; Granit, 1955). However, with compressions of the size used no change of length or bending of the axon could be detected within the error of measurement. Attention was then moved to clearly observed changes in the radial dispositions of the lamellae (Text-fig. 9), and the displacements referred to in this paper are all of this type. Radial displacements near each end of a lamella were sometimes measured as well as the central ones shown in the diagram and all were found to behave in the same way. By measuring displacements from photographs taken at successive intervals of time the time course of the displacement of any particular lamella from its rest position could be calculated. Text-fig. 10*a* shows such a time course due to a compression of 90 μ with a mean velocity of 35 μ/msec. It will be seen that the rise is sharp and that a well-defined peak occurs as the compression flattens out, this peak falling to a level which remains as long as the compression is held on. The figure shows this for three lamellae within the corpuscle, the numbers on the curves showing the ratio of the radius of the chosen lamella to the radius of the outer capsule. It may be noted here that, for the lamella nearest the core, the peak falls to zero and that the total displacement of this lamella is of a short, pulse-like form. It is shown in the next section that this form may, by an analytical procedure, be separated from the displacements of all the lamellae.



Text-fig. 10. Lamellar displacements at three positions within a corpuscle (see inset). *a*, total displacement as recorded; *b*, equivalent static displacement (see text and Text-fig. 12); *c*, dynamic component of displacement (see text and Text-fig. 12). Note ordinate starting levels have been shifted for clarity. Compression began at $t=0$ and was that shown in Text-fig. 9. The numbers on the curves are the ratios (radius of chosen lamella): (radius of outer capsule). The tolerance on all curves is $\pm 1{\cdot}5\,\mu$.

Analysis of the response

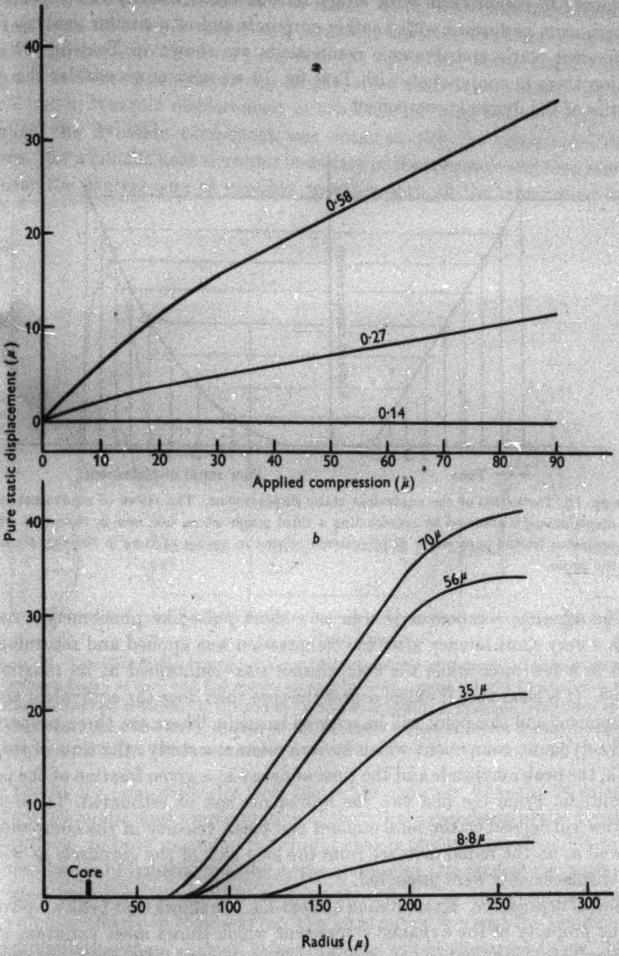
The displacements described above were total displacements measured directly from photomicrographs taken during and after an applied compression. Since the corpuscle is rapidly adapting (Gray & Malcolm, 1950) and is known to display a critical slope for excitation (Gray & Matthews, 1951), it was of interest to analyse the results in such a way as to be able to show the difference of the mechanical behaviour of the corpuscle when compressed either with zero or with finite velocity. An analogy to this difference in, perhaps, more familiar terms is the difference of the response of an electrical network before and after all the reactive elements have been removed, leaving only pure resistances. Accordingly, the total displacement was separated into two components, an 'equivalent static component' and a 'dynamic component'. The former was obtained (by a process described below) from photomicrographs taken during calibration, where compression was applied in stages and a photograph taken a few seconds after each advance. The displacements thus obtained were, clearly, independent of time and related only to the amount of compression applied and to the particular lamella chosen. They have been called the 'pure static component' and may be regarded as a steady-state component using the analogy suggested above, that is to say, the same as the recorded response after a sufficiently long time. Text-fig. 11*a* shows this component, as a function of compression, for three different lamellae within the same corpuscle, and it will be seen that the lamella nearest the core was not displaced even when 90μ compression was applied. Fig. 11*b* shows more clearly the radial dependence of this component, from a lamella near the outer capsule to one as close to the core as was resolvable.

Derivation of the equivalent static component

If any time-dependent compression (of which the simple linearly rising one used in this work is an example) is applied to the corpuscle and if the response of the corpuscle to the same amount of compression applied at zero velocity (that is, so slowly that transients are negligible) is known, then it is easy to construct a curve of displacement against time which refers only to the pure static response described above and yet is related to the time course of the applied compression. Text-fig. 12 shows this diagrammatically. The common ordinate represents compression, the left-hand abscissa time, and the right-hand abscissa displacement. The left-hand curve is determined only by the shape of the compression pulse applied and is that recorded by the transducer, while the right-hand curve is the same as Text-fig. 11*a* for one lamella. If a third graph is now drawn relating values of time 1, 2, 3, 4, ..., on the left-hand graph with values of displacement 1′, 2′, 3′, 4′, ..., on the right-hand graph, the resultant curve will show the displacement of the chosen lamella at any instant as if the amount of compression at that instant had been produced at zero velocity. This curve has been defined as the equivalent static component and Text-fig. 10*b* shows its form for three lamellae within the same corpuscle. As a general rule it more or less follows the shape of the compression pulse (clearly, it would follow it exactly if the stress–strain relation for the corpuscle were linear) so that the peaks appearing in the curves II and III represent the consequence of using a slightly peaked compression pulse. Curve I shows zero displacement and represents a lamella near the core.

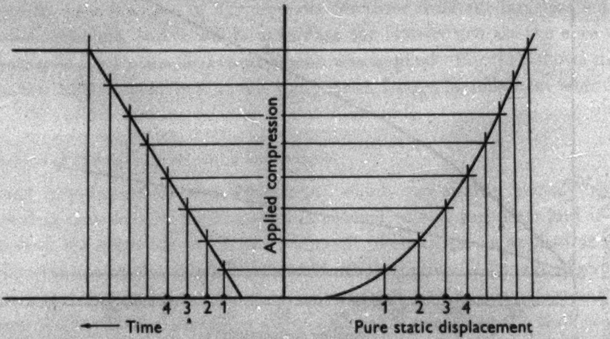
Derivation of the dynamic component and its properties

If the curves in Text-fig. 10*b* are subtracted from those in Text-fig. 10*a* the results are the curves in Text-fig. 10*c*. They have been defined as the dynamic component of displacement for any chosen lamella and in view of the physical significance of the two components from which they are derived they may be seen as one representation of the difference of the mechanical response of the



Text-fig. 11. The pure static component as a function of radius and compression. *a*, as a function of compression; the numbers on the curves are the ratios (radius of chosen lamella) : (radius of outer capsule); *b*, as a function of radius; the numbers on the curves are the total compression applied.

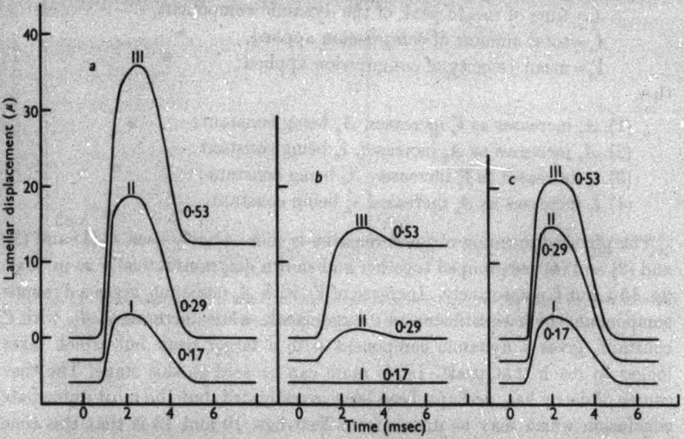
corpuscle to compression with either zero or finite velocity. The results of experiments performed with another corpuscle and of a similar analysis into equivalent static and dynamic components are shown in Text-fig. 13 and taking them in conjunction with Text-fig. 10 we may now consider the properties of the dynamic component.



Text-fig. 12. Derivation of the equivalent static displacement. The curve of equivalent static displacement is obtained by constructing a third graph whose abscissa is time and whose ordinates are the pure static displacements related to values of time in the way shown in the figure.

The dynamic component is seen as a short pulse-like phenomenon rising with a very short latency after the compression was applied and returning to zero in a few msec while the compression was maintained at its maximum value. It is clear that it exists nearer the core than does the equivalent static component and this point will be referred to again. There are three properties of the dynamic component which invite systematic study; the time of rise to peak, the peak amplitude and the time of decay to a given fraction of the peak amplitude. From the first two the rate of rise can be estimated. These properties will depend on the total amount and mean velocity of the compression as well as on the radial distance from the long axis of the corpuscle at which the displacements were measured.

Radial dependence. Examination of Text-fig. 13*c* shows that peak amplitude is the property of the dynamic component which shows most variation with radius. This is not, perhaps, so clear from Text-fig. 10*c*, where peak amplitude seems little different in the three lamellae measured; however, in the experiments from which Text-fig. 10 was derived resolution near the core was not good enough to allow measurement as near the core as in the experiments to which Text-fig. 13 refers. From the latter, although only three radii have been considered, two observations may be made. First, that peak amplitude decreases with decreasing radius and secondly, that the dynamic component persists further into the core than does the equivalent static displacement. Curve I in Text-fig. 13 shows this clearly, since there is zero static displacement but a dynamic displacement of the order of 10μ. To investigate this further, the dynamic component was obtained—by the process described above—for a lamella near the outer boundary of the corpuscle and for a lamella as near the central core as possible, together with all the intervening ones.



Text-fig. 13. Lamellar displacements at three positions within a corpuscle. The applied compression began at $t=0$ and had maximum amplitude 62μ, mean velocity $27\ \mu$/msec. *a*, total displacement as recorded; *b*, equivalent static displacement; *c*, dynamic component of displacement. The numbers on the curves are the ratios (radius of chosen lamella):(radius of outer capsule). The tolerance on all curves is $\pm 1{\cdot}5\mu$.

The results for two corpuscles were plotted as shown in Text-fig. 14 so as to give a more continuous representation of the radial dependence of the dynamic component. In both examples the abrupt change of slope corresponded to an abrupt change of lamellar spacing in the corpuscles, the spacing decreasing as the core was approached. The attenuation of the dynamic component is shown quite clearly in these curves; the justification for, and the significance of, extrapolating them at small values of radius will be discussed below. They may be compared with Text-fig. 11*b* showing the radial dependence of the pure static component.

Dependence on applied compression. Sufficient evidence has not yet been accumulated for an unequivocal relation to be given between any of the properties of the dynamic component and the applied compression. The time of rise to peak and the peak amplitude, and hence the rate of rise, of the dynamic component in terms of either the total amount or the mean velocity of compression would seem to be of immediate interest. To find these relationships it is clearly necessary to make all the measurements on one preparation, varying velocity, for example, while keeping the total amount of compression constant, or vice versa. This has been done in a few experiments only and the preliminary results so obtained have made possible four qualitative statements:

If A_r = peak amplitude of the dynamic component,
t_r = time of rise to peak of the dynamic component,
A_s = total amount of compression applied,
V_s = mean velocity of compression applied;

then

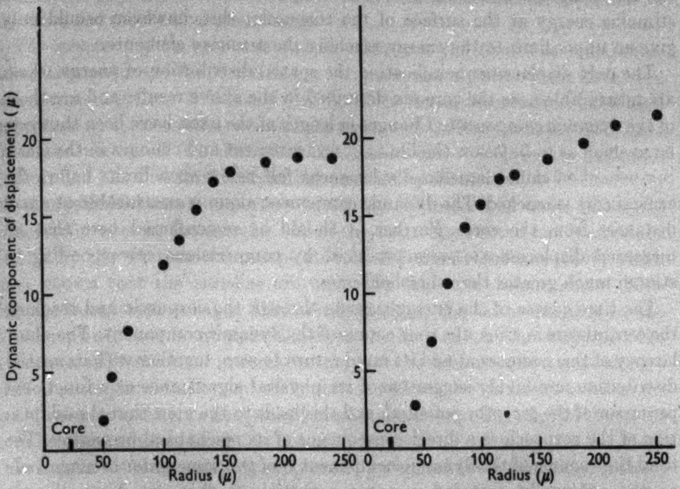
(1) A_r increases as V_s increases, A_s being constant;
(2) A_r increases as A_s increases, V_s being constant;
(3) t_r decreases as V_s increases, A_s being constant;
(4) t_r increases as A_s increases, V_s being constant.

The physical meaning of these remarks is more clearly seen if (1) and (3), and (2) and (4) are grouped together and shown diagrammatically as in Text-fig. 15*a* and *b* respectively. Increase of V_s, with A_s constant, gives a dynamic component which rises faster to a larger peak, while increase of A_s, with V_s constant, gives a dynamic component with a larger peak but which takes longer to reach that peak. Little more can be said at this stage. The time course of decay has, perhaps, been least investigated, but the most immediate conclusion which may be drawn from Text-figs. 10 and 13 is that this time course is short, being not more than 10 msec and often less.

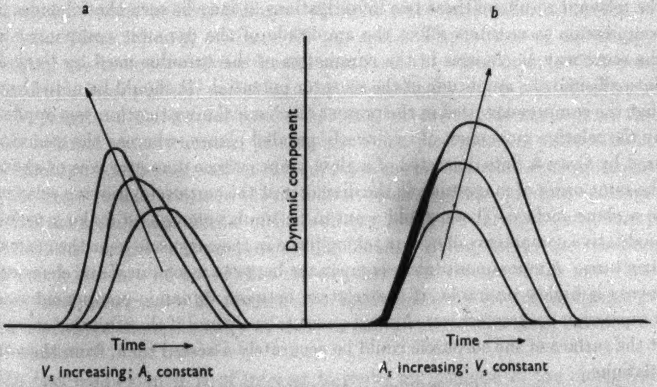
DISCUSSION

The reality of the receptor potential as an intermediate stage in the production of the action potential in sensory nerves seems, now, to be fairly well established. Indeed, the evidence from the three mechanoreceptors quoted in the Introduction is unequivocal and direct. Attempts must now be made to link the receptor potential with the stimulus and, for mechanoreceptors, part of this must be a mechanical link. The results of this work present evidence for the nature of this link behaving as a transformer of energy from the external (stimulus) form to the form in which it arrives at the sensitive elements of the receptor.

The significance of the dynamic component. The passage of energy through the corpuscle may conveniently be analysed in terms of three properties; the absolute quantity of energy involved, its spatial distribution, and its time course. The absolute quantity of energy passing through the corpuscle has not yet been measured. If the internal pressure and the surface force of



Text-fig. 14. Peak amplitude of the dynamic component as a function of radius for two different corpuscles. Abscissa, radius; ordinate, peak amplitude of dynamic component.



Text-fig. 15. Changes in the dynamic component with applied compression. Common ordinate, dynamic component; abscissae, time. *a*, change of dynamic component when V_s, the mean velocity of compression, increased, the total amount, A_s, remaining constant; *b*, change of dynamic component when A_s increased, V_s remaining constant. In both graphs the arrow shows the change resulting from an increase in either compression parameter.

the outer capsule were known it would be possible, at least, to calculate the stimulus energy at the surface of the corpuscle; this, however, would only give an upper limit to the energy reaching the sensitive elements.

The only displacements indicating the spatial distribution of energy which are measurable near the core are described in the above results and are those of the dynamic component. Changes in length of the axon have been shown to be so small as to be below the limits of measurement and changes in the static component of radial lamellar displacement fall below such limits before the central core is reached. The dynamic component alone is measurable at small distances from the core. Further, it should be remembered here that all measured displacements were produced by compressions corresponding to stimuli much greater than threshold ones.

The time course of the energy passing through the corpuscle and reaching the central core is, thus, the time course of the dynamic component. The short latency of this component and its rapid return to zero, together with its spatial distribution, are highly suggestive of its physical significance as a functional precursor of the receptor potential, and this leads to the view that the adaptation of the corpuscle is a direct consequence of its mechanical properties. The total time course of the dynamic component is of the same order of magnitude as the externally recorded receptor potential; further, its description in terms of compression parameters as shown in Text-fig. 15*a* and *b* agrees, within its qualitative limit, with the findings of Gray & Sato (1953) which related the stimulus directly with the receptor potential. For, by comparing the relevant results of these two investigations, it may be seen that changes in compression parameters affect the amplitude of the dynamic component in the same way as changes in the parameters of the stimulus used by Gray & Sato affected the amplitude of the receptor potential. It should be noted here that the compression used in the present mechanical investigation was applied by the relative movement of two nearly parallel planes, whereas the stimulus used by Gray & Sato consisted of a glass probe (whose diameter was of about the same order of magnitude as the diameter of the corpuscle) moving relative to a plane surface. These would seem to be similar enough to make a useful qualitative comparison of events taking place in the corpuscle near the central structures. A more quantitative comparison between mechanical and electrical events is highly desirable; if the relation between dynamic component and compression parameters were known quantitatively and if the stimulus energy at the surface of the corpuscle could be accurately assessed then, from the two relations

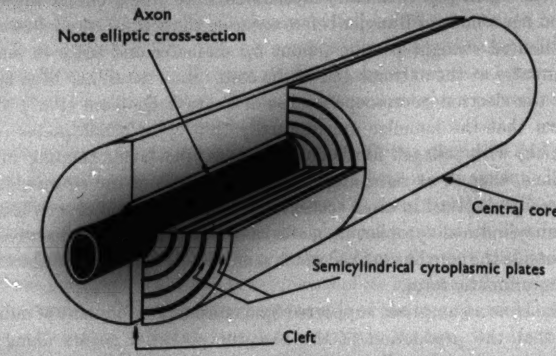
compression ... dynamic component
and stimulus ... receptor potential

the compression and stimulus could be eliminated and a quantitative comparison made between dynamic component and receptor potential. A better way, but technically much more difficult, would be simultaneous recording of electrical and mechanical phenomena.

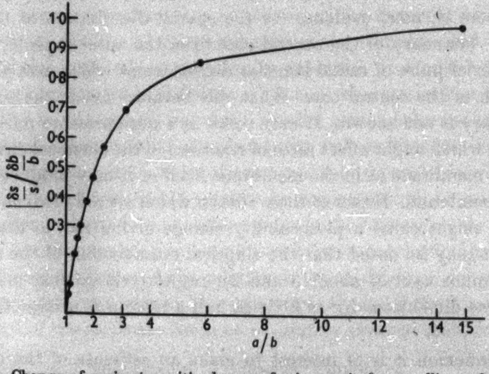
The structure of the corpuscle and its relation to function. The remarkable lamellar structure of the corpuscle has had many descriptions which have been summarized by Gray & Malcolm (1950). More recent observations have been concerned with the central core and the axon, and Quilliam & Sato (1955) have described the small (6μ diameter) myelinated fibre which enters the outer capsule and has a node of Ranvier before entering the central core followed by an unmyelinated straight portion (about 3μ diameter and 600μ in length) which bifurcates at the extreme end of the core. More detail has been added to this by the electron microscope studies of Pease & Quilliam (1957) which have shown that the lamellae are unusually thin cytoplasmic plates (150–500 mμ thick) with collagen fibrils on both sides, the latter running in the circular plane, that is, at right angles to the long axis of the corpuscle; the axon is usually elliptical in cross-section and lies with its major axis along a cleft of semicylindrical cytoplasmic plates of about the same thickness as the lamellae outside the core but packed much more closely. Text-fig. 16 shows the core in diagrammatic form.

It is a plausible assumption, supported by a considerable amount of indirect evidence, that the production of the receptor potential occurs along the unmyelinated section of the axon and that the result of a mechanical stimulus is a transient change of permeability (Gray, 1956). In the hydromechanical system of a fluid-filled laminated structure displayed by the corpuscle it is not obvious what interior changes will arise when external compression is applied. There is, now, evidence for the spatial distribution of the energy reaching the boundary of the central core from the outer capsule; it is contained in a brief pulse of radial lamellar displacement which acts along most of the length of the central core. What this becomes inside the core at the axon boundary is not known. It may result in a pure pressure pulse without deformation which might affect rates of reactions in the chemical environment of the nerve membrane or in the membrane itself or it may produce deformation of the membrane. Either of these effects, which are only two out of many possibilities, might cause a permeability change giving rise to the receptor potential. It may be noted that the elliptical cross-section of the nerve has major and minor axes of about 3 and 2μ respectively so that a change in either of these dimensions by as little as half a micron is a large fraction of the whole.

In this connexion it is of interest to make an estimate of the change of perimeter of the axon which would occur if the major or minor axis changed by a known amount. Since no change of length of the axon has been observed, and we may assume near-uniformity of cross-section of the axon, a change of minor axis, say, of this cross-section implies a change of perimeter and, thus, a change of surface area. The elliptical shape of the cross-section is then of considerable importance since it gives a non-zero change of perimeter for a small change of minor axis; small changes in the diameter of a circle give no change in perimeter. If we assume further that the cylinder is incompressible then there is no volume change on deformation and the problem is one of finding what fractional change of perimeter is produced for a given fractional


Axon
Note elliptic cross-section
Central core
Semicylindrical cytoplasmic plates
Cleft

Text-fig. 16. Diagrammatic summary of the electron microscope findings of Pease & Quilliam (1957). The detail is of the central core and axon showing the close packing of semicylindrical cytoplasmic plates and the position of the axon within the cleft



Text-fig. 17. Change of perimeter with change of minor axis for an ellipse of constant area. Abscissa, a/b; ordinate,

$$\left|\frac{\delta s}{s}\Big/\frac{\delta b}{b}\right|,$$

where a = semi-major axis; b = semi-minor axis; s = perimeter change of minor axis of an ellipse whose area is constant. Since the perimeter of an ellipse is not a simple function this involves computation of integrals whose integrands themselves contain elliptic integrals of the first kind. This has been done, therefore, for a few points only to cover the range between a circle and an ellipse having a major: minor axis ratio of 16:1 and Text-fig. 17 shows the result. It may be seen that for a major: minor axis ratio of 1·5:1 (which was the average figure found by Quilliam) the fractional change of perimeter is nearly one third of the fractional change of minor axis. If some assumption were made connecting change of perimeter and, hence, of surface area with certain changes of permeability it might be possible to calculate the receptor potential so that its value of peak amplitude, for example, could be compared with that found in practice.

There are several properties of the corpuscle which have not yet been investigated in terms of the displacements described in this paper; in particular, the 'off-response' (Gray & Malcolm, 1950) and changes of adaptation (Loewenstein, 1956). It is hoped that these will be considered in the more systematic study of the relation between dynamic displacement and receptor potential which is now being pursued.

SUMMARY

1. A technique has been described for investigating the response of the cat's mesenteric Pacinian corpuscles to applied compression. The method was largely an optical one involving photomicrography with a brief high-intensity source of illumination.

2. The spacing of the lamellae between the central core and the outer capsule was found to be logarithmic, abrupt changes occurring once or twice only within the same corpuscle.

3. During and after applied compressions of peak amplitude 19–90μ and of mean velocities 2·5–40μ/msec neither change of length nor bending of the axon was observed.

4. With the same range of compressions the total radial displacement of the lamellae could be separated into an 'equivalent static displacement' and a 'dynamic component of displacement'. The equivalent static displacement was derived from the pure static displacement which occurred when compression was applied at zero velocity, that is, so slowly that transients were negligible.

5. The pure static component decreased with decreasing radial distance and its attenuation could be roughly correlated with change of lamellar spacing.

6. The dynamic component showed less radial attenuation than the static and persisted nearer the central core. Resolution was not sufficient to describe movement of the core boundary or radial displacement of the axon within the core.

7. Some properties of the dynamic component were related qualitatively to the applied compression. If the total amount of compression were held

constant, increase in its mean velocity gave a dynamic component which rose faster to a large peak; while if the mean velocity were held constant, increase in the total amount of compression gave a dynamic component with a larger peak which, however, took longer to reach that peak.

8. The significance of the dynamic component is discussed, with the conclusion that the rapid adaptation of the corpuscle is a direct consequence of its mechanical properties.

9. The production of the receptor potential by radial deformation of the axon, and the results of calculations relating the latter to a change of surface area of the axon, are discussed in conjunction with recent electron microscope studies of the axon and the central core.

I wish to thank Dr J. A. B. Gray for continued encouragement and advice throughout this work, and Dr J. Diamond for many stimulating discussions and helpful criticisms. Acknowledgement is due also to the University of London Central Research Fund for some of the apparatus used in this work.

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EXPLANATION OF PLATE

Photomicrograph of the corpuscle from which Text-fig. 7 was derived. The abrupt change of spacing at the tenth lamella out from the core may be seen.

(*Facing p.* 218)

J. Physiol. (1958) 141, 219–232

THE ACTION OF INHIBITORS ON THE FACILITATED HEXOSE TRANSFER SYSTEM IN ERYTHROCYTES

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There is now a great deal of evidence indicating that hexoses do not cross the red cell membrane by simple diffusion but by a facilitated mechanism. The results of LeFevre (1948, 1954), LeFevre & LeFevre (1952), Widdas (1951, 1952, 1954), Wilbrandt & Rosenberg (1951) and Rosenberg & Wilbrandt (1955) show that the process behaves as a saturation mechanism, depends on steric configuration and shows competitive and inhibitory effects. It is suggested by most workers that the first stage in the transfer involves the formation of a complex between the hexose and a component of the membrane. Such a reaction is implicit in the kinetic treatments of the above authors (see Bowyer, 1957).

Kinetic treatments show that there are at least three possible mechanisms which would fit the available experimental data:

(1) The carrier system of Widdas (1952) which is equivalent to the non-enzymic carrier of Rosenberg & Wilbrandt (1955). This model postulates the presence of carriers in the membrane which pass backwards and forwards across the membrane owing to thermal agitation, irrespective of whether they are saturated with hexose or not. The carrier could be lipid, lipoprotein, or protein in nature.

(2) The enzymic carrier system of Rosenberg & Wilbrandt (1955). This is similar to the carrier system above except that the formation and dissociation of the carrier complex is catalysed by an enzyme (or enzymes) present in the membrane, which is itself immobile.

(3) The polar 'creep' hypothesis (Bowyer & Widdas, 1956*a*) which is equivalent to the polar pore system suggested for glycerol transfer by Stein & Danielli (1956). In this case no mobile membrane component is postulated, but it is suggested that a series of hexose adsorbing sites exists through the membrane along a polar region from outside to inside. Only one hexose adsorbing site is exposed to each interface. The hexose would then cross the

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membrane, after initial complex formation at the interface, by a chain or 'creep' process along the hexose adsorbing sites. Such a polar region could rather loosely be termed a pore. The possibility that the pore is occupied by a protein helix has been suggested by Burgen (1957).

The above possible mechanisms will be referred to as carrier, enzyme and pore theories respectively.

Until recently the kinetic approach has failed to distinguish between the three possible mechanisms and, in fact, it is difficult to visualize evidence which could not be interpreted in more than one way. Therefore other approaches have been combined with kinetics in the hope that further light may be thrown on the nature of the transfer process.

It has been known for some time that glucose transfer in the human red cell is inhibited by mercuric chloride, *p*-chloromercuribenzoate, iodine and phlorrhizin (LeFevre, 1948), phloretin (Wilbrandt, 1950), polyphloretin phosphate (Wilbrandt & Rosenberg, 1950), the lachrymators allyl*iso*thiocyanate, bromacetophenone and chloropicrine (Wilbrandt, 1954), 2:4-dinitrofluorobenzene (DNFB), dinitrochlorobenzene and dinitrobromobenzene and urethane (Bowyer, 1954; Bowyer & Widdas, 1956*a*).

The mercurial compounds react with sulphydryl (—SH) groups and also with amino (—NH_2) groups, though less readily with the latter (Edsall, 1954). However, the phenyl mercurials are unspecific in their action and have a general depressing action on enzyme activity (Smalt, Kreke & Cook, 1957). It is suggested that they may act by causing denaturation.

Dinitrofluorobenzene reacts irreversibly with —SH, —NH_2 groups and with phenolic hydroxyl. When red cells are inhibited with DNFB, glucose permeability cannot be restored by repeated washing with alcoholic saline, indicating that the inhibition is due to an irreversible action of the inhibitor.

The inhibiting action of phlorrhizin is not understood. The inhibitor is known to have an effect on the energy-supplying reactions of the cell, but these appear to play no part in the glucose transfer. Wilbrandt (1954) suggests a possible inhibition of phosphatase. He points out that lachrymators are known to inhibit hexokinase, but ATP and Mg^{2+}, which accelerate hexokinase activity, did not accelerate glucose transfer in the red cell (Wilbrandt, 1954). Moreover, the action of these enzyme inhibitors is non-specific and possibly indicates the participation of a protein in the transfer mechanism rather than a specific enzyme.

Using polyphloretin phosphate labelled with ^{32}P, Wilbrandt (1954) showed that the highly polymerized molecule did not penetrate the red cell and suggested that the action of the inhibitor was on the outer surface of the cell membrane. He also showed that polyphloretin phosphate inhibited the exit of glucose more than the entry. From this he concluded that there were two enzymes on the outer surface, one of which was inhibited by polyphloretin phosphate and preferentially involved in outward transport. More recent experiments by Wilbrandt, Frei & Rosenberg (1956) showed that the rate constant for the entry of glucose into human red cells was higher than that for exit (K_E entry $=7\times10^{-2}$; K_E exit $=4\times10^{-2}$).

The question has therefore arisen as to whether the transfer process is more complex than can be represented by present treatments, which presume a symmetrical transfer system, i.e. one in which rates of entry and exit should bear similar relationships to the concentrations of hexoses when only the direction of the concentration gradient is reversed. A real discrepancy between entry and exit rates as described by Wilbrandt *et al.* (1956) would be of importance in attempting to understand the kinetics of the transfer process and would affect the interpretation of inhibitor results.

The present paper therefore describes (*a*) further experiments to investigate differences in rate constants for entry and exit and (*b*) experiments with inhibitors of three different types:

(1) a competitive inhibitor, urethane, which can penetrate the red cell;
(2) a competitive inhibitor, polyphloretin phosphate, which cannot enter the cell;
(3) the irreversible inhibitor DNFB.

A preliminary account of part of this work has been given to the Physiological Society (Bowyer & Widdas, 1957).

METHODS

The apparatus used (Ørskov type described by Widdas (1953*a*)); the experimental technique and the method of analysing the results of entry experiments have been described previously (Widdas, 1954). Human erythrocytes were used throughout. For exit experiments cells previously equilibrated in a glucose medium were centrifuged and a fixed quantity (about 3 mm^3) of packed cells was resuspended in 0·2 ml. of the supernatant and added rapidly to 21 ml. buffered saline in the cuvette. The resuspension was found necessary in order to obtain a rapid dispersion of the cells so that their volume change could be followed more accurately. The diluted suspension only contributed a concentration of glucose of 0·05 mM to the outside medium, which was negligible compared with concentration inside the cells. This can be allowed for when necessary.

Solutions of inhibitors were made up in buffered isotonic saline. A stock solution of DNFB was prepared by dissolving 44·4 mg in 11·4 ml. ethanol before making up to 100 ml. with saline. Since DNFB is readily hydrolysed a fresh stock solution was made up for each experiment. This was diluted as required, the proportion of alcohol to buffered saline not being allowed to exceed 0·4 ml. in 21 ml., in order to eliminate any effect on the stability of the red cell suspensions in the cuvette or upon hexose transfer.

The polyphloretin phosphate used was obtained from Leo, Hälsingborg, and had an estimated molecular weight of 15,000.

RESULTS

The entry and exit rates for the transport of glucose across the human red cell membrane were measured over the range 38–76 mM. The results were analysed as in the previous paper (Widdas, 1954) using the approximation that for glucose,

$$\text{transfer rate} = K\phi\left(\frac{1}{C_1} - \frac{1}{C_0}\right), \tag{1}$$

where C_0 and C_1 refer to the outside and inside concentration of hexose, ϕ is the equilibrium constant, and K is a constant. This approximation is derived from the more precise equation

$$\text{transfer rate} = K\left(\frac{C_0}{C_0+\phi} - \frac{C_1}{C_1+\phi}\right), \tag{2}$$

on the assumption that both C_0 and C_1 are very large relative to ϕ (see Widdas, 1951; Wilbrandt & Rosenberg, 1951).

In order to calculate the transfer rate from equation (2) it is necessary to insert numerical values for ϕ, and these have been taken from Widdas (1953*b*, 1954) and LeFevre (1954).

The rate constants for exit and entry, calculated from equations (1) and (2), are compared in Fig. 1.

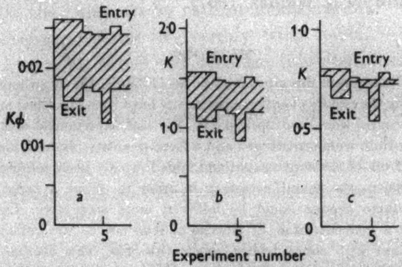

Fig. 1. Rate constants for entry and exit of glucose. Human erythrocytes; temp. 37° C. Exit experiments carried out from 76 to 38 mM glucose; entry from 38 to 76 mM. Results calculated (*a*) from eqn. 1, (*b*) from eqn. 2 ($\phi_g = 7$ mM), (*c*) from eqn. 2 ($\phi_g = 17$ mM).

The results from equation (1) give rate constants for glucose entry, which exceed those for exit by 40%. Wilbrandt *et al.* (1956) reported a difference of the order of 75%. The use of equation (2) reduces the difference to about 12%, showing that the errors due to the approximation fall unevenly on the equations for entry and exit.

In the case of sorbose, the rate constants for exit and entry are not calculated from the approximate equation (1), as the equilibrium constant (ϕ_s) is high compared with the concentrations. Errors arising from the use of equation (1) are thus not introduced in the sorbose results. Using a diffusion type equation (Widdas, 1954), rate constants for exit and entry differ by 15%, which is the same order of magnitude as experimental error.

It therefore appears justifiable to look at the results of inhibitor experiments in terms of a symmetrical transfer system.

Theoretical considerations

Since the experimental conditions were varied and the processes of inhibition might operate in different ways, it was desirable to express all the results initially in a way which made no assumptions as to the underlying processes.

If P_0 represents any rate constant of the hexose transfer system in the absence of inhibitor, and P_1 the same constant when inhibitor is present, then the fraction $\frac{P_0 - P_1}{P_0}$ represents the fractional inhibition of the transfer system independent of the units used. This treatment could be applied to experiments in which the inhibition of sorbose entry, glucose entry, or glucose exit into a glucose-free medium was measured, although the rate constants found in the three types of experiment have different units. The fractional inhibition should be some function of inhibitor and hexose concentrations, and can conveniently be compared with theoretical predictions.

Competitive inhibitors

If two species of substrate are capable of reaction with the membrane component, the state of adsorption equilibrium of each species separately may be defined by

$$\theta_A = \frac{x}{1+x} \quad \text{and} \quad \theta_B = \frac{y}{1+y}$$

where θ is the fraction of the complex

$$x = \frac{C_A}{\phi_A}, \quad y = \frac{C_B}{\phi_B},$$

and C and ϕ refer to the appropriate concentrations and equilibrium constants respectively.

In a competitive system

$$\theta_A = \frac{x}{1+x+y} \quad \text{and} \quad \theta_B = \frac{y}{1+x+y}.$$

In the treatments which follow, species A is the hexose of which the rate of penetration is measured, and species B is the inhibitor. Subscripts 1 and 2 are used to indicate outside and inside the cell respectively.

The basic assumption in the pore hypothesis is that specific sites in the two interfaces correspond and that transfer can only occur if one site is occupied by hexose and the other is not. The inward flux is thus proportional to the probability that a site in interface S_1 is saturated (θ_1) multiplied by the probability that the corresponding site in S_2 is unsaturated $(1-\theta_2)$. Similarly, outward flux will be proportional to the product $\theta_2(1-\theta_1)$.

The net transfer may be represented as

$$\underset{S_1 \rightarrow S_2}{\text{Transfer}} = K\{\theta_1(1-\theta_2) - \theta_2(1-\theta_1)\}, \tag{3}$$

which in the simplest experimental conditions reduces to

$$\underset{S_1 \rightarrow S_2}{\text{Transfer}} = K(\theta_1 - \theta_2), \qquad (4$$

which is identical to the simplest form of the carrier kinetics. Inserting the conditions appropriate to competitive inhibition, however, we have

$$\underset{S_1\rightarrow S_2}{\text{Transfer}}=K\left(\frac{x_1}{1+x_1+y_1}\times\frac{1}{1+x_2+y_2}-\frac{x_2}{1+x_2+y_2}\times\frac{1}{1+x_1+y_1}\right), \tag{5}$$

and theoretical dependence of the fractional inhibition on values of x and y for different experimental conditions can be derived from equation (5).

A carrier system may be regarded as made up of many individual units. A cycle of activity for one unit would result in transfer if the carrier crossed from S_1 to S_2 saturated with hexose and returned from S_2 to S_1 unsaturated. To a first approximation the probability of this event would be proportional to $\theta_1(1-\theta_2)$. Similarly, the probability of hexose transfer from S_2 to S_1 would be $\theta_2(1-\theta_1)$, and net transfer would be given by equation (3).

In a simple carrier model it is also necessary to allow for the possibility of exchange. Thus it would be sufficient for net transfer of one sugar if the 'carriers' returned unsaturated or saturated with the other sugar.

This can be represented by

$$\underset{S_1\rightarrow S_2}{\text{Transfer}}=K\left(\frac{x_1}{1+x_1+y_1}\times\frac{(1+y_2)}{1+x_2+y_2}-\frac{x_2}{1+x_2+y_2}\times\frac{(1+y_1)}{1+x_1+y_1}\right) \tag{6}$$

Evaluation of x and y

In order to use the above equations, it is necessary to determine values of ϕ for glucose and the inhibitors so that concentrations can be expressed as multiples of the equilibrium concentration.

To do this the inhibition of sorbose entry by inhibitor or by glucose can be used (in this case glucose is treated as an inhibitor). There is considerable evidence (LeFevre, 1953; Widdas, 1953*b*, 1954) that the equilibrium constant for sorbose is high and that, for the concentration at which the experiments are carried out, $x < 0{\cdot}05$ and may be neglected without serious error.

With this approximation, equation (6) reduces to

$$\frac{P_0-P_1}{P_0}=\frac{y}{1+y};$$

that is, 50% inhibition will occur when $y=1$ and ϕ will equal the concentration of inhibitor at which sorbose entry is reduced to 50%.

Equation (5) reduces to

$$\frac{P_0-P_1}{P_0}=\frac{y(2+y)}{(1+y)^2},$$

and 50% inhibition occurs when $y=0{\cdot}414$; thus $\phi=2{\cdot}42\ C$, where C is the concentration at which sorbose entry is 50% inhibited. This would represent the case of an inhibitor which reacted with the glucose adsorbing site or carrier, but did not move through the membrane on the hexose system.

The value of ϕ obtained thus depends on the different approaches and assumptions made, but as equations (5) and (6) apply equally to a pore or carrier model, they have been used to predict fractional inhibitions which are compared with experimental results.

For this purpose equation (5) can be written in the form

$$\frac{P_0-P_1}{P_0}=\frac{y_1y_2+y_1(1+x_2)+y_2(1+x_1)}{(1+x_1+y_1)(1+x_2+y_2)}, \tag{7}$$

which can be further simplified when $y_1=y_2$ or when $x_1=0$, as in exit experiments.

If $y_2=0$, i.e. if the inhibitor cannot penetrate the cell, equation (7) becomes

$$\frac{P_0-P_1}{P_0}=\frac{y_1}{1+x_1+y_1} \tag{8}$$

and for exit experiments when $x_1=0$

$$\frac{P_0-P_1}{P_0}=\frac{y_1}{1+y_1}. \tag{9}$$

Inhibitor results

Competitive inhibition by urethane. The penetration of urethane into human red cells is very rapid. The rate of penetration is greater than that for glycerol and is not suitable for quantitative measurement by the methods used in these experiments. Tests with cells in which the hexose transfer had been inhibited more than 95% by DNFB still showed urethane entry too rapid to measure quantitatively and it may be presumed that the hexose system is not essential for urethane penetration of red cells. The evidence for the reaction of urethane with the hexose transfer system is based solely on its behaviour as an inhibitor.

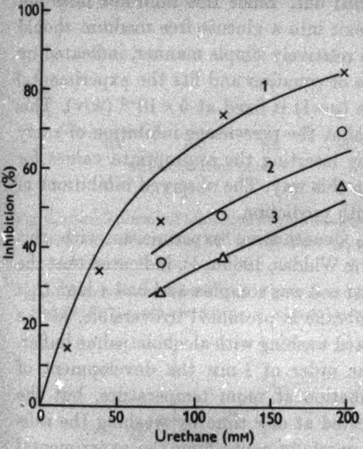

Fig. 2

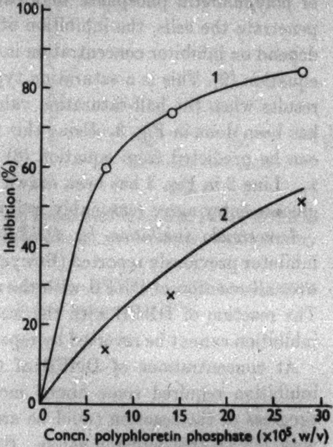

Fig. 3

Fig. 2. Inhibition of hexose transfer by increasing concentrations of urethane. ×, sorbose entry; ○, glucose exit; Δ, glucose entry. Curve 1 drawn according to eqn. 5 to fit the experimental results (y = urethane, mM/137). Curves 2 and 3 are lines predicted from eqn. 7 using same values of y. Values of x obtained from $x = C_g/\phi_g$ where $\phi_g = 7$ mM.

Fig. 3. Inhibition by polyphloretin phosphate. ○, glucose exit; ×, glucose entry. Curve 1 drawn according to eqn. 9 to fit experimental results (y = concn. polyphloretin phosphate (w/v) (5×10^{-5})). Curve 2: predicted line drawn from eqn. 8 using same values of y. Values of x obtained from $x = C_g/\phi_g$, where $\phi_g = 7$ mM.

Its rapid penetration, however, makes it justifiable to assume that it will maintain an equilibrium in which its concentration is equal inside and outside the cell and its inhibitory activity agrees with predictions made on this basis.

In Fig. 2 are shown the percentage inhibitions of sorbose entry, glucose exit and glucose entry as the urethane concentration is varied. For a given urethane concentration it is found that inhibition of sorbose entry > inhibition of glucose exit > inhibition of glucose entry. This is the order expected on theoretical grounds for a competitive inhibitor and is quantitatively of the right order when the results are treated as described in the Theoretical section. The lines 2 and 3 of Fig. 2 are the predicted values using values for ϕ_g and ϕ_u derived from sorbose inhibition. It is assumed that the inhibitor does not move through the membrane on the hexose transfer system but competitively inhibits at each interface.

Competitive inhibition by polyphloretin phosphate. Two types of experiment were performed on each cell suspension, which gave respectively the inhibition of rates of exit and entry. Repeated experiments at different concentrations of polyphloretin phosphate were carried out. Since this inhibitor does not penetrate the cells, the inhibition of exit into a glucose-free medium should depend on inhibitor concentration in a relatively simple manner, indicated by equation (9). This is a saturation type of equation and fits the experimental results when the half-saturation value ($y=1$) is fixed at 5×10^{-5} (w/v). This has been done in Fig. 3. Using this value, the percentage inhibition of entry can be predicted from equation (8) by inserting the appropriate values for x_1. Line 2 in Fig. 3 has been drawn in this way. The observed inhibitions of glucose entry agree reasonably well with prediction.

Irreversible inhibition by DNFB. Quantitative experiments with this inhibitor previously reported (Bowyer & Widdas, 1956*a*, *b*), indicated that the over-all reaction of DNFB with the red cell was complex and had a high Q_{10}. The reaction of DNFB with the membrane is presumed irreversible, as the inhibition cannot be reversed by repeated washing with alcoholic saline buffer.

At concentrations of DNFB of the order of 1 mM the development of inhibition required some hours' incubation at room temperature, but the progress of the reaction could be arrested at any time by washing the cells with DNFB-free buffered saline. By carefully controlling the experimental conditions, reasonably reproducible fractional inhibitions were obtained. However, entry inhibition developed more slowly than inhibition of exit (see Fig. 4).

It has been postulated (Stein & Danielli, 1956) that in the human red cell glycerol is transferred by a facilitated diffusion, but independently of the hexose system. A comparison of the inhibition of the entry of glucose and of glycerol by DNFB in human red cells also showed a difference in the rate of development of inhibition. Glycerol transfer was affected more slowly. This is shown in Fig. 5.

In the course of this study it was observed that when cells were incubated with DNFB in the presence of glucose, the inhibiting reaction proceeded more rapidly. This effect was investigated with varying glucose concentrations, with the result shown in Fig. 6. The shape of this curve suggests a saturation phenomenon, and the glucose concentration giving half the maximum effect

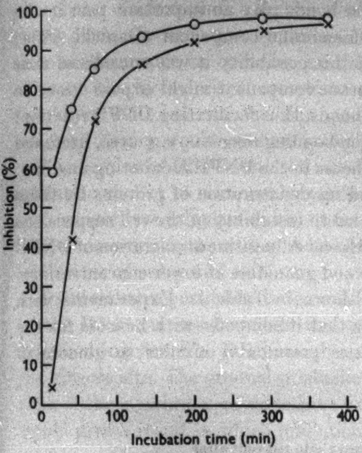

Fig. 4

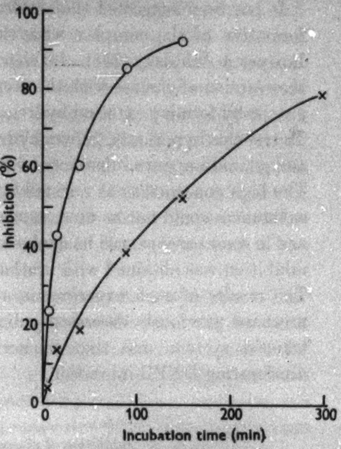

Fig. 5

Fig. 4. Development of inhibition of glucose entry and exit on incubation with 1·4 mM DNFB at 20·5° C in presence of 100 mM glucose. ○, glucose exit; ×, glucose entry. Exit and entry measured at 37° C.

Fig. 5. Development of inhibition of glucose and glycerol entry on incubation with 1·4 mM DNFB at 24° C. ○, glucose entry; ×, glycerol entry. Entries measured at 27° C.

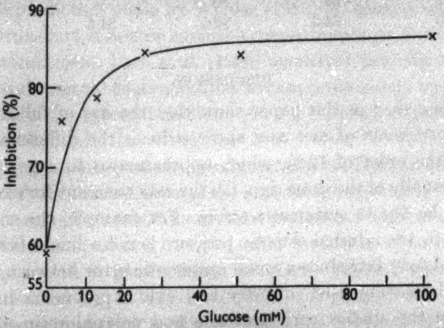

Fig. 6. Acceleration of inhibitory reaction of DNFB on glucose entry into red cells by incubation in the presence of glucose. Incubation at 20° C for 132 min with 1·4 mM DNFB.

corresponds reasonably with the concentration of glucose which half saturates the hexose transfer system at this temperature. This effect is the opposite of what might be expected if glucose and DNFB were reacting with the same component of the membrane, and its full interpretation is not yet clear.

It has been suggested that hydrogen bonds play an important part in the formation of the complex with the membrane component (Danielli, 1954; Bowyer & Widdas, 1955). In view of this possibility it was considered that the reaction of glucose with the membrane component might expose reactive groups by forming external hydrogen bonds, thus facilitating DNFB reaction. To test this hypothesis, known hydrogen bonding reagents, e.g. urea, urethane and guanidine, were substituted for glucose in the DNFB incubating medium. The high concentrations normally used for denaturation of proteins by these substances could not be used, as they led to instability of the cell suspensions and in some cases caused haemolysis. However, positive acceleration of DNFB inhibition was obtained with urethane and guanidine at lower concentrations. The results of such experiments are shown in Table 1. Experiments with urethane previously described indicate that it competes with hexoses for the transfer system, and thus its action is presumably similar to glucose in accelerating DNFB inhibition.

TABLE 1

Inhibition of glucose entry into red cells after incubation at 20° C with 1·4 mM DNFB

	Inhibition (%)			
		Incubated in presence of		
Time of incubation (min)	Control	Glucose 50 mM	Urethane 100 mM	Guanidine 100 mM
132	53	80	—	80
180	64·5	92·5	84·5	—

DISCUSSION

The results described in this paper show that the use of fuller equations in analysing experiments of exit and entry reduces the difference in the rate constants to the order of 12%, which approximates to experimental error. This error is usually of the same sign, i.e. the rate constant for exit < for entry, but this may be due to systematic errors. For example, the contribution of haemoglobin to the internal osmotic pressure is not a linear function of concentration and may introduce a small systematic error between conditions of shrinking and swelling used in entry and exit experiments in the Ørskov technique. In the studies with urethane and polyphloretin phosphate the greater degree of glucose exit inhibition can be accounted for on the basis of a symmetrical system, provided the reactions at both interfaces are regarded as essential to the transfer process and are provided for in the kinetic treatments.

These results do not disprove the enzyme theory, which gives similar kinetics. It is unnecessary to suggest separate enzymes for the formation and hydrolysis of the complexes and that these are differently affected by polyphloretin phosphate. In fact, such a system would be a case of Maxwell's Demon and would cause accumulation of glucose. This has not been reported. Further, since urethane acts on both the outer and the inner interfaces, a preferential effect on the hydrolysis of complexes would cancel out and entry and exit should be inhibited equally. The increased inhibition by urethane of exit relative to entry would need to be explained in a different way.

The reaction of DNFB with the membrane appears to be irreversible, as the inhibition cannot be removed by washing. Therefore DNFB cannot be acting as a competitive inhibitor in the normal manner. However, if the inhibition is non-competitive there should be no difference in the development of the inhibitions of entry and exit.

An alternative possibility is that DNFB causes 'internal competition'. That is, the DNFB is not bound to the specific glucose-adsorbing site but to a neighbouring site. The DNB–membrane component could then compete for the glucose site. The gradual production of an 'internal competitor' on both sides of the membrane would behave like increasing amounts of urethane, and would give a result in which inhibition of entry developed more slowly than inhibition of exit.

That the inhibition by DNFB of glycerol entry develops more slowly than the inhibition of glucose entry might be due to a difference in chemical reactivity of the two mechanisms, and offers additional evidence of the independence of the respective transfer systems.

Recent results by Park, Post, Kalman, Wright, Johnson & Morgan (1956) confirmed the prediction made by Widdas (1952) of 'counter transport'. This involves the transport of hexose against a concentration gradient under certain competitive conditions. Park *et al.* (1956) incubated red cells in xylose until the external and internal concentrations were approximately equal. They then added glucose to the external medium and the internal xylose concentration fell, i.e. xylose had left the cell against a concentration gradient as glucose entered. Such an 'uphill' transfer is only temporary. The prediction was made on the basis of the carrier hypothesis, and the phenomenon can readily be explained in terms of a mobile membrane component with which xylose and glucose combine competitively at the outer interface, while initially xylose alone combines with it at the inner interface. Similar results have been obtained by Rosenberg & Wilbrandt (1957) for glucose and mannose. The 'counter transport' cannot easily be explained in terms of a non-mobile membrane component if it is postulated that only one site is exposed to the medium at each interface (see below).

The possible role of enzymes in the transfer process might be re-examined in view of the increased knowledge of transferase activity of hydrolytic enzymes (Morton, 1955). Morton has shown that during the hydrolysis of a phosphate ester molecule (the donor) by an enzyme, the phosphate group is transferred to a second molecule (the acceptor); e.g. when phenyl phosphate is hydrolysed by acid phosphatase, primary alcohols, glycols, glycerol and glucose can act as acceptors. Hydrolysis by alkaline phosphatase transfers phosphate to glucose, fructose and glycerol, while hydrolysis by hexose diphosphatase transfers phosphate to sorbose. It is possible for the same enzyme to hydrolyse the phosphate ester produced, and so the original molecule is reformed.

Phosphatases are known to be present in regions where transfer of glucose takes place, e.g. proximal kidney tubule, intestinal mucosa and red cells, and it has long been postulated that they play some part in the transfer mechanism (Danielli, 1953).

In the light of recent work it is possible to attempt a synthesis of the enzyme, carrier and pore models. The basic postulate would be an enzyme molecule (e.g. we might consider a phosphatase) at each interface. The molecule, following Morton's suggestions, would have a donor site and an acceptor site. The first stage in transfer would be the attachment of glucose to the acceptor site. It is necessary to postulate the existence of a donor molecule attached to the donor site, e.g. it could be a phosphate molecule similar to Morton's phenyl phosphate, or a physiological compound. When this molecule is hydrolysed the glucose is esterified and thereby displaced from the site. At least two models would then be reasonable to explain transfer through the membrane:

(1) The glucose ester could be transported across the lipid membrane by diffusion. At the inner interface the molecule would become attached to the donor site on the enzyme and be hydrolysed. The glucose would be liberated to the inside of the cell and the phosphate, for example, would become attached to another acceptor molecule. An essential step in the transfer is the return of the (phosphate) compound to the outer interface. Thus the complete circulation of one phosphate or similar molecule is part of the transfer and in this respect the system could be called a 'carrier' system. No discontinuity of the non-polar membrane need be presumed, and this model is essentially similar to Rosenberg and Wilbrandt's enzymic carrier transport.

(2) The enzyme sites may be located at discontinuities in the lipid membrane (polar pores) and the glucose phosphate could either diffuse or be transferred directly (or via intermediate sites) to a donor site at the inner interface. The same circulation of phosphate would be necessary either across the membrane or in local circuits between intermediate sites in the pore. This model would be adequate to explain 'counter transport'.

SUMMARY

1. The entry and exit rate constants for the transport of glucose across the human red cell membrane have been determined over a range of concentrations from 38 to 76 mM. Although the constant for entry exceeds that for exit, it has been shown that the major part of this difference is due to approximations in the equations used, and that to postulate an asymmetrical transfer system is unjustifiable.

2. A symmetrical system of transfer can also account for different degrees of entry and exit inhibition observed in experiments with inhibitors such as urethane and polyphloretin phosphate, provided that the reaction at both interfaces is allowed for in the kinetic treatment.

3. The inhibitory reaction of dinitrofluorobenzene (DNFB) with the red cell appears to be irreversible and the development of inhibition to be accelerated by glucose. During development of inhibition on incubation with DNFB exit is affected more than entry. It is difficult to account for these results in terms of a non-competitive inhibition. The possibility of an 'internal competitor' has been discussed.

4. Some proposed models of transfer have been discussed in the light of the present and recent work.

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MUSCULAR EXERCISE ON MOUNT EVEREST

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Although mountain climbing was one of the first kinds of muscular exercise to interest physiologists, little work appears to have been done in this field since the early years of this century. Between 1900 and 1909 Zuntz and Durig and their colleagues studied the respiratory exchanges in men walking uphill and on the level at various altitudes from near sea level to 14,000 ft. on Monte Rosa (Zuntz, Loewy, Müller & Caspari, 1906; Durig, 1911).

Zuntz measured the rise in ventilation and decline in oxygen consumption at high altitude in subjects walking at the pace they naturally adopt on Alpine tours, and he showed that the efficiency of mountain climbing was greatly influenced by the nature of the ground and by the experience and state of training of the subject.

Douglas, Haldane, Henderson & Schneider (1913), in metabolic studies on men walking uphill on Pike's Peak, showed that under controlled conditions a given amount of work required the same amount of oxygen at 14,000 ft. as at sea level. They also confirmed the effect of the terrain on the efficiency of walking—showing that walking on rough grass required about 30% more oxygen than walking at the same pace on a smooth surface.

During the International Physiological Expedition to the Andes in 1935, Christensen (1937) studied the effect of altitude up to 17,500 ft. on men working on a bicycle ergometer. His results confirmed the findings of Douglas *et al.* (1913) that the efficiency of muscular exercise was independent of altitude, and further showed that although lung ventilation in litres (BTPS) was greatly increased at high altitudes, ventilation in litres (STPD) was independent of altitude over a wide range of altitudes and rates of work. (BTPS: body temperature, observed pressure, saturated with water vapour; STPD: standard temperature and pressure, dry gases.) At the highest station (17,500 ft.) he observed a decline in maximum ventilation (STPD) and heart rate during exercise, and, since the heart rate corresponded with that expected for the observed rate of work and oxygen consumption at lower altitudes, he concluded that the heart was not strained and that the limiting factor in muscular work at altitude was the maximum ventilation possible (Christensen & Forbes, 1937).

In a study of men acclimatized in a low-pressure chamber, Houston & Riley (1947) took samples of arterial blood from their subjects during work and showed that arterial oxygen tension was reduced even at low intensities of work compared with the resting state, and that the alveolar-arterial oxygen gradient was increased, thus demonstrating by direct experiment that the hypoxia of altitude is increased during muscular exercise.

The work described here was begun on a training expedition to Mount Cho Oyu in 1952 in order to obtain information on the respiratory exchanges of mountaineers under Himalayan conditions for use in the designing of oxygen equipment for the 1953 Everest Expedition, and it was later continued on that expedition itself. Measurements were made of oxygen consumption and ventilation during muscular work at varying altitudes. The step test was used as a convenient form of standardized exercise, but studies were also made on men climbing at their natural pace at various altitudes, and during short periods of exhausting work. Maximum voluntary ventilation was also measured. The results of these observations are compared with results obtained in treadmill experiments at sea level and in walking uphill at low altitudes in this country.

These investigations, although fragmentary, have confirmed the basic principles just described which were established at lower altitudes by previous workers and, in addition, have brought to light some new aspects of muscular exercise at high altitude which could not have been anticipated from the earlier work.

METHODS

Subjects

Details of the ages, heights and weights of members of the Mount Cho Oyu and Everest Expeditions who acted as subjects for the experiments are given in Table 1. The weights are those recorded at the beginning of the respective expeditions and can be regarded as the subjects' usual body weights. Weights given in the tables of results are those recorded at the time of the experiments, and in many cases are considerably below the usual weight as a result of the sojourn at high altitude. An altitude conversion table has been added to Table 1.

Apparatus

Pulmonary ventilation was measured either with a Max–Planck Institute respiration meter (Orsini & Passmore, 1951) or by a modified bag method which has been described previously (Pugh, 1953).

The Max–Planck respirometer was found to be unsuitable for exercise studies above 12,000 ft. owing to high respiratory pressures and leakage at the slide valves at rates of ventilation over 60 l./min. Another difficulty was the unreliability of temperature measurements when the respirometer was exposed to the sun.

The bag method avoided the use of a gas-meter altogether. Instead of collecting expired air over a standard time interval, one measured the time required to fill the bag to capacity. The bags were of light rubberized fabric, weighing about 2 lb. (0·9 kg) and having a capacity of 250–300 l. They were used either with a low-resistance valve unit and mouth-piece, or without valves, in which case the subject exhaled directly into the bag, controlling the neck manually. The bag was filled to 20–30 mm Hg above atmospheric pressure and closed with a surgical clamp. The pressure and temperature of the contained gas were measured, and the volume read off on a pressure–volume chart prepared for each bag before the expedition, and checked subsequently. At high rates of ventilation, pressure built up rapidly over the last few breaths, but comparison with other methods has shown that this does not affect the respiratory minute volume significantly (Pugh, 1953). When no respiratory valves are used the minute volume may be underestimated owing to incomplete collection of each breath and leakage due to the neck of the bag not being grasped with sufficient force. In practice this does not appear to happen to any significant extent; nor does there appear to be any interference with ventilation, at least in trained subjects, since normal ventilation equivalents for oxygen are found.

TABLE 1. Physical characteristics of members of the Mount Cho Oyu and Everest Expeditions who acted as subjects

Subject	Age (years)	Weight (kg)	Height (cm)
T.D.B.	28	88·5	185
R.C.	29	59·4	173
R.C.E.	33	69·9	174
E.P.H.	33	84·8	187
G.P.	42	78·0	183
C.S.	39	87·1	188
T.S.	34	95·2	194
M.P.W.	27	74·8	178
M.H.W.	27	71·7	174
C.W.	32	69·9	182

Altitude conversion table

ft.	m	ft.	m	ft.	m
1000	305	2100	640	3000	914
1200	366	2200	671	4000	1219
1300	396	2300	701	5000	1524
1400	427	2400	732	6000	1829
1500	457	2500	762	7000	2133
1600	488	2600	792	8000	2438
1700	518	2700	823	9000	2743
1800	548	2800	853	10,000	3048
2000	610	2900	884		

Gas samples were either collected over mercury and analysed on the spot, or stored in sealed glass ampoules and brought back to England. Analyses were performed on a Micro-Scholander gas analyser (Scholander, 1947). It was found that up to 13 analyses a day could be done at 20,000 ft. The difference between duplicates seldom exceeded 0·1% of O_2 or CO_2, and subsequent checking of the calculations revealed few mistakes, probably because a pocket calculating machine was used rather than a slide-rule. The chief source of error in using the Micro-Scholander apparatus under these conditions arose from fluctuations in ambient temperature. Sometimes the temperature of the water-bath had to be adjusted, and check analyses of atmospheric air performed, several times a day. The method of drawing gas samples into evacuated 50 ml. glass ampoules was similar to that used by Greene on Mount Everest in 1933 (Greene, 1934). On the expedition to Mount Cho Oyu the ampoules were sealed in the flame of a Primus stove. As this proved a difficult and uncertain procedure, a cylinder of butane fitted with a Bunsen burner was taken to Mount Everest and used successfully up to 24,000 ft.

Experimental procedure

Step tests. Oxygen consumption was determined on one subject (G.P.) during step tests at 11,600 ft. and 20,000 ft. on the Cho Oyu Expedition using the Max–Planck respirometer, and at 6600 ft. and 20,500 ft. on the Everest Expedition by the bag method. The tests consisted of 15-min periods of stepping on and off a box at a constant rate of either 20 or 30 steps/min. Boxes 6, 8½ and 10 in. (15, 22, 25 cm) high were used on different occasions. The total weight raised varied according to the subject's weight at the time, the amount of clothing worn, and the weight of the apparatus. Expired air was collected during the last 5 min of the 15-min work period. Control values at sea level were taken from some thirty laboratory experiments on the same subject spread over a period of 2 yr, in which step rates of 20, 24 and 30 steps/min and heights of 6, 10, 12 and 20 in. (15, 25, 30, 51 cm) were used in various combinations. Work rates were calculated from the equation

$$WR = W \times S \times H,$$

where WR is the work rate (kg.m/min), W the total weight raised (kg), S the number of steps per minute and H the height of the step (m). This of course ignores the step down but is comparable to calculation of the climbing rate on the mountain.

Walking uphill in the Himalayas. On the expedition to Cho Oyu respiratory measurements were made on three subjects (T.D.B., R.C. and C.S.) at 15,500 and 20,000 ft. Particulars of the time spent at altitude and the nature of the terrain are given in some detail because of their obvious bearing on the results. The experiments at 15,500 ft. were carried out during a rest period following the unsuccessful assault on the mountain 2 months after the expedition began. The party had already been 3 days at 15,500 ft. when the experiments were begun, and were feeling thoroughly rested and fit; before that they had spent 4 weeks continuously at heights between 17,000 and 22,500 ft. The slope chosen for the experiments was a typical Himalayan hill-side, consisting of fine scree sparsely covered with juniper scrub and having a gradient of approximately 1 in 3·5. The subjects had to pick their way and to traverse from side to side. The procedure adopted was as follows. The subjects walked uphill at their natural pace without any control of the work rate other than the feeling of effort. They were simply asked to climb at their habitual pace for the prevailing conditions. After suitable practice, each subject made two test ascents of approximately 350 ft. over the same ground, the height climbed being measured with an aneroid altimeter. The nose was closed with a clip and the bag carried by its neck in front of the body. After the first 5 min an observer, suitably posted, met the subject and released the clamp on the neck of the bag. Without stopping, the subject then began to exhale directly into the bag and continued so doing until the bag was full, at which point he was met by a second observer who closed the bag with another clamp. A third observer recorded the times. The experiments at 20,000 ft. were begun about a week later, 3 days after coming up from the camp at 15,500 ft. They were carried out on a steep snow slope up which a zig-zag track had been laid. The snow surface froze hard at night and did not begin to thaw out until about 11 a.m. The experiments on C.S. and R.C. were completed before thawing occurred, but there was some softening of the surface in one of the experiments on T.D.B. The height of the slope, which was measured trigonometrically, was 285 ft. Before reaching it the subjects had about 500 yards (457 m) to walk over flat ground. The method of collecting expired air was the same as that used at 15,500 ft. Climbing rates were measured on ascents to the top of the hill without collection of expired air. In the light of experience gained at the lower altitudes this was considered preferable to attempting to measure ventilation and climbing rate on the same ascent using the altimeter.

In 1953 observations were added on two other subjects (M.P.W. and G.P.) climbing at 6000 ft. during the approach march to Mount Everest. The subjects had been marching 5 hr a day at altitudes varying from 4000 to 9000 ft. for the previous 2 weeks, and before that had spent a week in Katmandu at 4000 ft. The measurements were made in the ordinary course of the day's march on a 3000 ft. climb on a path. The method of collecting expired air was the same as that just described. Heights were measured by altimeter.

Exhausting work of short duration. Maximum oxygen consumption and ventilation during short periods of exhausting exercise were studied on the expedition to Mount Everest. Experiments were done on M.P.W., C.W. and G.P. between 7000 and 8000 ft. during the approach march; on M.P.W., E.P.H. and three Sherpas at the main base at Thyangboche (13,000 ft.), and on M.P.W., T.S. and G.P. in the West Cwm on Mount Everest at either 20,500 ft. or 21,200 ft. The observations at 7000 and 8000 ft. were made about 14 days after leaving sea level; those at 13,000 ft. on the third day after returning from a 3-day visit to 18,000 ft. 5 weeks after leaving sea level, and those at 20,500 ft. and 21,200 ft. 10–12 weeks after leaving sea level, the last 4 weeks of which had been spent above 18,000 ft. The procedure was similar to that used in the experiments on sustained climbing, except that the subjects walked uphill at the fastest pace they could maintain for 5 min. The collection of expired air was begun after they had been climbing for 3 min. In the West Cwm on Mount Everest no suitable slopes were available for experiments of this kind, so the subjects performed maximum exercise by step-climbing on a 10 in. box at the rate of 30 steps/min. Two of the three subjects, however, could not keep up this rate for as long as 5 min, and in their case the rate was either reduced to 20 steps/min or sampling was begun after 2 min instead of after 3 min. The experiments also differed from those conducted at lower altitude in that a low-resistance valve unit was used instead of breathing directly into the bag, the reason being that they formed part of a series of experiments on the effects of supplementary oxygen for which valves were essential.

Maximum voluntary ventilation. The 15-sec maximum voluntary ventilation (M.V.V. test; also known as the maximum breathing capacity, M.B.C.; Comroe, Forster, DuBois, Briscoe & Carlson, 1955) was measured, at the suggestion of Dr John Cotes, at the beginning of the expedition and again at 20,500–21,200 ft. on three subjects (M.P.W., C.W. and G.P.). A further test was performed on G.P. at the end of the expedition. The tests were carried out in the sitting position without control of the respiratory rate. The subject, wearing a nose clip, breathed into a low-resistance valve unit, having duplicate J valves in parallel, which was connected to a light-weight bag. After a single deep inspiration he inserted the mouthpiece and breathed in and out as deeply and quickly as possible for 15 sec. The time was measured from the beginning of the first inspiration to the end of the expiration nearest to 15 sec. The volume of expired air collected was measured by passing the contents of the bag through the Max–Planck Institute respirometer. Three tests were performed on each subject at each altitude.

Treadmill experiments at sea level. Maximum pulmonary ventilation and oxygen consumption were measured on four members of the Everest team by Drs D. J. C. Cunningham, R. G. Bannister and R. S. McCormack in Professor C. G. Douglas's laboratory at Oxford. M. P.W., M.W. and G.P. were studied before the expedition left, and E.P.H. 4 weeks after the expedition arrived back in England. None of the subjects was particularly fit at the time, especially E.P.H. who was still under weight.

The subjects ran for two or more periods of 10 min with rests for recovery between each period. The speed of the treadmill was 6·5 miles (10·5 km)/hr. In the first or preliminary period the treadmill was horizontal. In the second and third periods (which were the test periods) it was tilted to a gradient of 1 in 10. For E.P.H., however, the treadmill was set at 1 in 11, because he did not feel fit to run to complete exhaustion. Expired air was collected by the bag method after 3 min of running, and samples were analysed on a Haldane gas analysis apparatus. Five minutes after each work period blood samples were taken for the determination of lactic acid, using the method of Barker & Summerson (1941).

Walking uphill at low altitude. In these experiments the subjects walked uphill on a standard course near Mount Snowdon in Wales. The oxygen consumption and ventilation rate were measured over a range of rates of work in order to provide results suitable for comparison with the results obtained on men climbing at their habitual pace at great altitudes. In most of these experiments volunteers from the R.A.F. acted as subjects as G.P. was the only member of the Everest team available. The normal duty of these men entailed walking in the neighbouring hills round a 7-mile (11·2 km) perimeter but although thoroughly accustomed to hill walking they were not trained mountaineers. The subjects climbed 350 ft. on a grass slope having a gradient of 1 in 5. Ascents were made at rates designated as slow, medium and fast, and an approximately constant pace was maintained during each ascent by regulating the number of steps per min. The respiratory apparatus used was the Integrating Motor Pneumotachograph (Wolff, 1956). The subjects made trial ascents wearing the apparatus until they had become thoroughly accustomed to the procedure.

RESULTS

Step tests

The results of step tests performed by G.P. at sea level and at various altitudes up to 20,500 ft. are presented in Table 2. Both the 15-min tests at moderate intensities of work and the 5-min tests at high intensity have been included in this table. The relation of oxygen consumption and ventilation to rate of work in the 15-min tests is shown in Fig. 1.

TABLE 2. The results of step tests on G.P. at various altitudes from sea level to 20,500 ft.

Altitude (ft.)	No of expts.	Step height (in.)	Step height (cm)	No. of steps/min	Rate of work (kg. m/min)	O_2 consumption (l./min)	Ventilation (l./min) STPD	Ventilation (l./min) BTPS	Ventilation equivalent (l./l. O_2) STPD	R.Q.
					Moderate work					
S.L.*	4	6	15	20	250	1·12	23·9	29·4	21·4	0·88
S.L.	4	6	15	30	375	1·50	32·6	40·2	21·7	0·91
S.L.	6	10	25	20	422	1·54	31·7	39·0	20·6	0·91
S.L.	2	12	30	20	486	1·61	37·8	46·5	23·5	0·94
S.L.	12	12	30	24	579	1·90	42·7	52·0	22·5	0·91
S.L.	4	10	25	30	633	2·20	47·4	58·7	21·6	0·93
6,600	2	8¾	22	20	335	1·37	25·4	38·6	18·6	0·89
11,500	1	6	15	20	254	1·32	20·7	40·4	15·7	0·90
20,000	1	6	15	20	238	1·19	19·6	52·4	16·5	0·88
20,500	2	10	25	20	403	1·42	28·1	75·9	19·8	1·09
					Exhausting work					
S.L.	4	20	51	30	1164	3·51	83·4	102·5	23·8	1·06
20,500	2	10	25	30	612	2·03	49·25	133·3	24·2	1·06

* In this and subsequent Tables S.L. = sea level

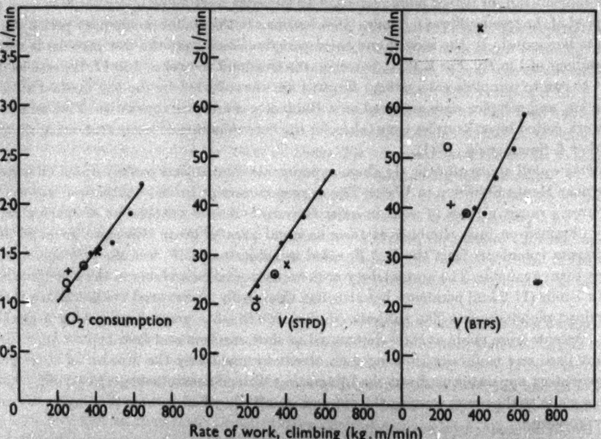

Fig. 1. Step tests at various altitudes from sea level to 20,500 ft. Relation of oxygen consumption and ventilation to the work of raising the body. Subject G.P. ●, sea level; ⊙, 6600 ft.; +, 11,500 ft.; ○, 20,000 ft.; ×, 20,500 ft.

At sea level oxygen consumption was proportional to the rate of work irrespective of the height or rate of stepping. This finding is consistent with the results of Passmore & Thomson (1950) when their data are plotted in the same fashion. Passmore & Thomson showed, however, that efficiency falls off when the rate for any given height of step is varied beyond certain limits. The results obtained at altitude are similar to the findings at sea level and conform to the principle established by previous workers (Douglas *et al.* 1913; Christensen, 1937; Houston & Riley, 1947) that the oxygen consumption for a given intensity of work is constant and independent of altitude over a wide range of rates of work.

The relation of ventilation to rate of work at sea level was linear over the range of rates of work studied. This was true whether ventilation was expressed in litres STPD or litres BTPS. At altitude, ventilation BTPS increased in such a way that ventilation STPD was virtually independent of altitude, although set at a slightly lower level (Fig. 1). Table 2 shows that the ventilation equivalent for oxygen (ventilation in l. STPD/l. STP of oxygen absorbed), also tended to be slightly below the sea-level values, particularly in the tests carried out with the Max–Planck respirometer. It has since been found that the high respiratory pressures set up in this apparatus at minute volumes over 40 l./min interfere with ventilation, giving rise to low values for the ventilation equivalent (H. S. Wolff, unpublished).

Taking this into account, the results, on the whole, support Christensen's (1937) finding that ventilation (STPD) for a given rate of work and the ventilation equivalent for oxygen are independent of altitude.

In the 5-min step tests the subject worked nearly to exhaustion, and it is safe to regard the oxygen consumption and ventilation as maximal for this form of work. The results show that at 20,500 ft. maximum oxygen consumption had fallen to a little over half the sea-level maximum, while ventilation (BTPS) was increased by 40 %.

The R.Q. in both sets of experiments were typical of moderate and exhausting work, respectively, and were not influenced by altitude except in the 15-min tests at 20,000 ft., where R.Q. exceeded unity, although the work was performed easily without strain. The most probable explanation is inaccurate gas analysis, since the samples were analysed on the spot at 20,500 ft. where analytical precision was not sufficient for reliable R.Q. determinations.

Observations on men climbing at their habitual pace

The experimental results are presented in detail in Table 3, and Fig. 2 illustrates the principal findings. Also included in Fig. 2 are the results obtained by Durnin (1955), in connexion with the 1953 Mount Everest Expedition, on men climbing at their natural pace near sea level on Ben Lomond.

The oxygen consumption maintained by the subjects studied at 15,500 and 20,000 ft was surprisingly high, the values observed being only slightly lower

Table 3. Oxygen consumption, ventilation and work rate in men climbing at their habitual pace at various altitudes

	Subject	Nude weight (kg)	Pulmonary ventilation (l./min)		Expired air		O_2 consumption		Ventilation equivalent (l./l. O_2) STPD	Rate of work, climbing (kg.m/min)	Rate of climbing	
			STPD	BTPS	O_2 (%)	CO_2 (%)	(l./min)	(ml./kg/min)			(ft./hr)	(m/hr)
Altitude 15,000 ft., Barometer 425 mm Hg (20. v. 52)	C.S.	75·0	35·2	80·3	15·35	5·54	1·97	26·3	17·9	—	—	—
		75·0	34·4	78·6	15·65	5·29	1·83	24·4	18·8	679	1620	494
	T.D.B.	81·8	45·0	102·7	16·57	4·17	1·99	24·3	22·6	786	1776	541
		81·8	45·6	104·1	16·71	4·38	1·91	23·4	23·9	738	1668	509
	R.C.	54·1	29·4	67·0	15·56	5·35	1·58	29·2	18·6	566	1860	566
		54·1	28·6	65·2	15·18	5·76	1·65	30·5	17·3	—	—	—
Altitude 20,000 ft., Barometer 370 mm Hg (27. v. 52)	C.S.	75·0	34·1	91·2	15·41	4·95	1·94	25·9	17·6	—	—	—
		75·0	30·0	80·2	15·41	4·95	1·72	23·0	17·5	—	—	—
		75·0	—	—	—	—	—	—	—	580	1352	411
	T.D.B.	81·8	46·4	123·9	16·39	4·01	2·18	26·7	21·3	—	—	—
		81·8	37·3	99·7	15·85	4·31	1·98	24·2	18·9	—	—	—
		81·8	—	—	—	—	—	—	—	499	1109	338
	R.C.	54·1	26·9	71·8	16·04	4·81	1·33	24·6	20·2	—	—	—
		54·1	29·3	78·4	15·91	5·09	1·48	27·4	19·8	—	—	—
		54·1	—	—	—	—	—	—	—	436	1391	423
		54·1	27·0	72·1	15·75	4·55	1·45	26·8	18·6	393	1283	391
Altitude 6,000 ft., Barometer 611 mm Hg (18. iii. 53)	G.P.	77·3	38·0	58·1	15·09	5·59	2·25	29·1	16·9	1037	2500	762
	M.P.W.	75·4	35·4	54·1	15·54	5·53	1·90	25·2	18·6	880	2100	640

than those found in climbers on Ben Lomond. Very high levels of ventilation (BTPS) were observed at these altitudes and the differences between individual values were greatly magnified. Ventilation (STPD), on the other hand, was virtually independent of altitude and followed the same trend as the oxygen consumption. The ventilation equivalents for oxygen were fairly uniform, the values at all altitudes being within the range reported in studies at sea level (Åstrand, 1952*a*).

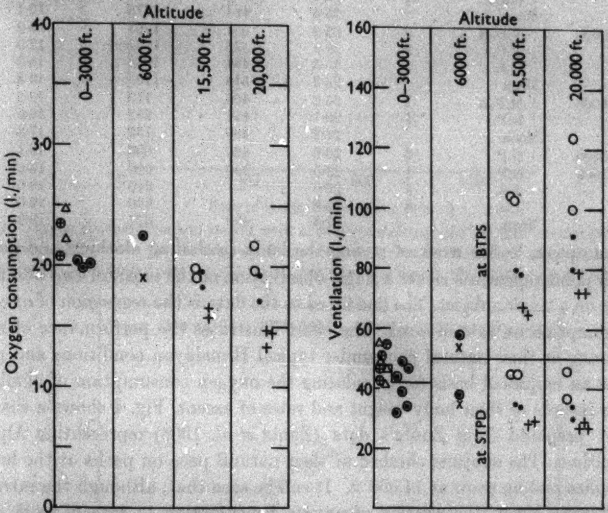

Fig. 2. Oxygen consumption and ventilation in acclimatized men climbing at their habitual pace at various altitudes. Plotted points represent single observations. Subjects: T.D.B., ○; R.C., +; C.S., ●; G.P., ⊙; M.P.W., ×; A.S., △; J.B., ⊕.

The individual values for the three subjects studied at 15,500 and 20,000 ft. illustrate the consistency of results that can be obtained when subjects are allowed to work at their natural habitual pace. The rather wide variation between individuals was largely due to differences in body size, and is greatly reduced by expressing the results in terms of unit body weight (Table 4).

The average rates of climbing in the experiments at 15,500 and 20,000 ft. were, respectively, 23 and 44 % less than the average Himalayan rate up to 6000 ft., whereas the corresponding rates for oxygen consumption were 5 and 10 % less at 15,500 and 20,000 ft. than up to 6000 ft. This means that climbing at great altitudes was less efficient than climbing nearer sea level.

In Fig. 3 the results have been plotted to show the relation of total oxygen

Table 4. Mean values for oxygen consumption and ventilation and the ventilation equivalent for oxygen in men walking uphill at their natural pace (observations on subjects J.B. and A.S. were made by Durnin in 1955 on Ben Lomond)

Altitude (ft.)	Subject	No. of expts.	O_2 consumption (ml./kg/min)	Ventilation (ml./kg/min) STPD	Ventilation (ml./kg/min) BTPS	Ventilation equivalent (l./l. O_2) STPD
20,000	T.D.B.	2	25·4	512	1367	20·1
	R.C.	3	26·3	513	1326	19·6
	C.S.	2	24·4	428	1143	17·5
	Mean		25·4	484	1278	19·1
15,500	T.D.B.	2	23·8	554	1264	23·2
	R.C.	2	29·9	535	1222	17·9
	C.S.	2	25·3	464	1059	18·3
	Mean		26·3	518	1182	19·8
6000	M.P.W.	1	25·2	469	718	18·6
	G.P.	1	29·1	491	752	16·9
	Mean		27·2	480	735	17·8
3500	G.P.	2	26·6	429	605	16·1
1000	G.P.	1	25·0	490	620	19·6
	A.S.	8	29·0	—	640	18·6
	J.B.	8	25·9	—	620	20·0
	Mean		27·5	490	627	19·3

consumption to the work of raising the body (including clothing and gear). Each point represents either a single observation or the mean of two observations on a single subject. The line fitted to the data is the regression of oxygen consumption on rate of work. The chart illustrates the performance of men climbing at their natural pace under typical Himalayan conditions and provides an empirical basis for calculating the oxygen consumption of climbers from records of their body weight and rates of ascent. Fig. 4 shows a similar chart prepared from Zuntz's data (Zuntz *et al.* 1906) representing Alpine conditions. The subjects climbed at their natural pace on paths at the lower altitudes and on snow at 14,000 ft. It can be seen that, although the rates of work were lower, the relation of oxygen consumption to rate of work was similar to that observed in the Himalayas.

Maximum oxygen consumption and maximum ventilation in exhausting work of short duration

The complete results are given in Table 5, and Table 6 provides a comparison with the work of other authors.

In the treadmill experiments at sea level the oxygen consumption of members of the Everest team were comparable with the results of Robinson (1938–39) on subjects who had no previous experience of running on the treadmill and who were not trained athletes. They are considerably below the mean values obtained by Åstrand (1952*b*) on well trained subjects accustomed to this type of exercise, and are much inferior to the existing values for first-class track athletes quoted by Robinson (1938–39) and the more recent figures reported by Åstrand (1955).

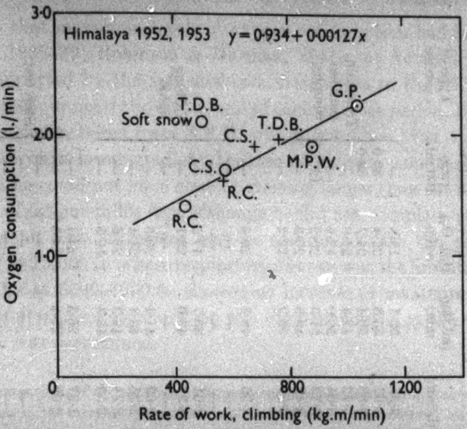

Fig. 3. Oxygen consumption and rate of work in subjects walking uphill at their normal pace at various altitudes in the Himalayas. Points represent data on individual subjects at each altitude. ⊙, 6000 ft., path; +, 15,500 ft., fine scree and bushes; ○, 20,000 ft., hard snow.

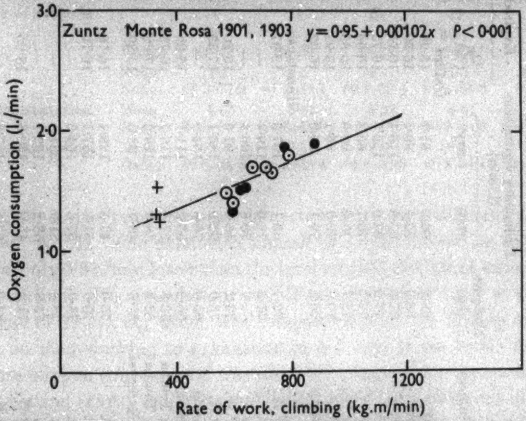

Fig. 4. Oxygen consumption and work rate in subjects walking uphill at their normal pace at various altitudes in the Alps (Zuntz *et al.* 1906). Points represent data on individual subjects at each altitude. ●, 2000 ft., path; ⊙, 6500 ft., rough stony path; +, 14,000 ft., hard snow.

The maximum ventilation did not show the extreme respiratory stimulation found in exercise of higher intensity carried to exhaustion in 4–6 min, where the ventilation equivalent may rise to 30 l./l. O_2 (Åstrand, 1952*a*). The post-exercise blood-lactate values in three of the subjects were consistent with the

TABLE 5. Maximum oxygen consumption and ventilation during muscular exercise at various altitudes

Date	Altitude (ft.)	Barometer (mm Hg)	Subject	Nude wt. (kg)	Pulmonary ventilation (l./min)		Expired air		O$_2$ consumption		Ventilation equiv. (l./l. O$_2$) STPD	Blood lactate (mg/100 ml.)	Type of exercise
					STPD	BTPS	O$_2$ (%)	CO$_2$ (%)	(l./min)	(ml./kg/min)			
							Oxford						
7. i. 53	S.L.	759	M.W.	68·2	66·9	81·1	15·56	5·63	3·55	52·1	18·8	—	Treadmill gradient running
7. i. 53	S.L.	759	M.W.	68·2	82·1	99·5	16·30	5·31	3·67	53·8	22·4	86·0	
7. i. 53	S.L.	759	G.P.	77·0	95·8	116·1	16·75	4·63	3·88	50·4	24·7	89·0	
7. i. 53	S.L.	759	G.P.	77·0	93·0	112·7	16·75	4·87	3·72	48·3	25·0	—	
29. i. 53	S.L.	763	M.P.W.	72·7	70·7	85·2	15·66	5·79	3·64	50·0	19·4	—	
29. i. 53	S.L.	763	M.P.W.	72·7	75·4	90·9	15·85	5·55	3·73	51·3	20·2	120	
28. vii. 53	S.L.	754	E.P.H.	70·2	70·2	85·7	15·92	5·47	3·43	48·9	20·5	—	
28. vii. 53	S.L.	754	E.P.H.	70·2	79·1	96·6	16·32	5·10	3·57	50·9	22·2	—	
							Himalayas						
19. iii. 53	6600	603	M.P.W.	72·7	69·1	107·3	15·97	5·46	3·35	46·1	20·6	—	Walking uphill
19. iii. 53	6600	596	G.P.	77·3	76·0	119·4	—	—	—	—	—	—	
20. iii. 53	6000	617	C.W.	—	70·6	106·8	—	—	—	—	—	—	
29. iii. 53	13,200	479	M.P.W.	73·2	45·8	91·5	15·20	5·93	2·61	35·7	17·5	—	
8. iv. 53	13,200	476	E.P.H.	75·0	92·0	185·1	17·33	3·86	3·27	43·6	28·1	—	
8. iv. 53	13,200	476	Tensing	65·0	76·8	154·5	—	—	—	—	—	—	
8. iv. 53	13,200	476	Tensing	65·0	55·2	111·1	15·96	5·39	2·69	41·4	20·5	—	
8. iv. 53	13,200	476	Ang Temba	52·3	57·5	115·7	16·79	4·33	2·36	45·1	24·4	—	
8. iv. 53	13,200	476	Ang Temba	52·3	58·8	118·2	17·09	4·19	2·21	42·3	26·6	—	
8. iv. 53	13,200	476	Da Namgyel	51·8	48·7	98·0	15·80	5·19	2·50	48·3	19·5	—	
21. v. 53	20,500	364	G.P.	75·0	47·5	129·4	16·76	4·39	1·97	26·3	24·1	—	Step test
22. v. 53	20,500	368	G.P.	75·0	51·0	137·1	16·79	4·42	2·09	27·9	24·4	—	
25. v. 53	21,200	349	G.P.	74·0	57·6	164·5	—	—	—	—	—	—	
26. v. 53	21,200	349	T.S.	76·8	81·0	231·5	—	—	—	—	—	—	
27. v. 53	21,200	349	T.S.	76·8	53·2	152·1	—	—	—	—	—	—	
31. v. 53	21,200	349	M.P.W.	71·4	45·8	130·8	18·05	3·29	1·28	17·9	35·8	—	
27. v. 52	20,000	370	T.D.B.	84·0	62·8	167·8	—	—	—	—	—	—	Walking uphill

assumption that maximum levels of oxygen consumption had been obtained (Robinson, 1938–39; Robinson & Harman, 1941*a*, *b*; Åstrand, 1952*c*), and this was supported by the subjects' own statements to the effect that they were exhausted, or nearly so, at the end of each exercise period. Blood samples were not, however, taken from E.P.H. who was studied after the expedition and who ran on a lower gradient because of his poor condition. However, E.P.H. said he could not have gone on running longer than the 10-min period on account of fatigue in his legs, although he did not complain of respiratory embarrassment. This statement may be contrasted with the experiment on this subject at 13,000 ft. where respiratory distress was the limiting factor.

The results at 6000–7000 ft. showed no increase in maximum ventilation, and maximum oxygen consumption was slightly reduced in the single subject on whom it was determined.

TABLE 6. Comparison of the maximum oxygen consumption observed in four members of the Everest team in the treadmill experiments at Oxford with those observed by other authors

		Mount Everest team	Untrained subjects (Robinson, 1938–39)		Well trained subjects (Åstrand, 1952*b*)	International class runners	
						(Åstrand, 1955)	(Robinson, 1938–39)
Number of subjects ...		4	11	10	17	3	5
Age (years)	Mean	32	—	—	29·7	—	—
	Range	27–42	20–29	32–38	27–33	—	—
Weight (kg)	Mean	71·2	72·5	79·4	71·5	64	—
	Range	68·2–77·0	61·2–84·4	60·2–93·5	61·9–86·6	60–66	—
Maximum O_2 consumption (l./min)	Mean	3·65	3·61	3·53	4·07	4·49	—
	Range	3·43–3·88	2·46–4·20	2·56–4·50	3·49–5·09	4·37–5·04	—
Maximum O_2 consumption (ml./kg/min)	Mean	50·1	47·1	52·8	57·1	74·0	69·02
	Range	48·3–53·8	36·4–55·4	44·6–62·5	48·1–64·1	72·4–76·6	63·3–81·5

At 13,000 ft. the two European subjects reacted very differently one from the other. E.P.H., who exhausted himself in 5 min, showed an oxygen consumption only 0·3 l./min lower than the level reached at Oxford when he was in poor condition. His ventilation was 185 l./min (BTPS) with a ventilation equivalent of 28 l./l. O_2, which was comparable with the finding of Åstrand (1952*a*) on men working to exhaustion in 4–6 min at sea level. In M.P.W. maximum oxygen consumption was some 30 % lower than the sea-level value and ventilation (BTPS) was comparable with the values observed in this subject at 6000 ft. and at sea level. M.P.W. did not, however, exert himself to quite the same degree as E.P.H.

The three Sherpas had maximum oxygen consumptions comparable with E.P.H. when allowance is made for differences in body weight, and these levels were reached without the extreme respiratory stimulation shown by E.P.H. One of the Sherpas was Tensing who was E.P.H.'s companion on the ascent to the summit of Mount Everest, and both the others carried loads to the South Col (25,850 ft.). The values for maximum oxygen consumption observed in the Sherpas, which ranged from 41 to 48 ml./kg/min, were well below the corresponding range of 50 to 60 ml./kg/min observed in well trained Europeans at sea level. Although born and raised at 13,000 ft., these men had been resident in Darjeeling and had come from there to join the expedition in Katmandu. Hence they were not acclimatized to the same degree as permanent residents at 13,000 ft. would have been.

In the experiments done in the West Cwm on Mount Everest, in which a step test was used instead of walking uphill, not all the subjects were able to complete the required exercise of 5-min step-climbing on a box 10 in. high 30 times/min.

At 20,500 ft. G.P. could just manage the 5-min test at this rate. His oxygen consumption and ventilation (BTPS) were 2·0 l./min and 133 l./min respectively (mean of two tests), the rate of positive work being 544 kg/min. The oxygen consumption corresponding to this rate of work at sea level was 1·9 l./min (Fig. 1). At 21,200 ft. this subject became very dyspnoeic and collection of expired air was begun after 2 min, showing a ventilation of 160 l./min. Oxygen consumption was not measured but can be assumed to have been less than 2·0 l./min. M.P.W. was unable to finish the test at 30 steps/min at 21,200 ft. His rate gradually fell to about 20 steps/min and at the end of 5 min he subsided exhausted on the box. He said afterwards that his main difficulty had been intolerable dyspnoea. The results of this test showed a ventilation of 131 l./min (BTPS) and an oxygen consumption of 1·3 l./min. Assuming the same efficiency as G.P., M.P.W.'s calculated oxygen requirements for steady work at 30 steps/min and 20 steps/min were 1·90 and 1·45 l./min respectively. The third subject, T.S., was also unable to complete the test, and had to stop after 4 min on account of dyspnoea and weakness. His ventilation was found to be 231·5 l./min (BTPS), which was the highest rate recorded on the Expedition even in maximum voluntary ventilation tests. In an experiment on the following day, at 20 steps/min, his ventilation was 152 l./min (BTPS). The performance of subjects T.S. and M.P.W. in these tests was much below expectation, and it was thought that they were suffering from high altitude deterioration.

In none of these subjects was there any evidence of circulatory failure. They recovered rapidly after exercise. No rise in central venous pressure or lung crepitations were observed. The radial pulse was difficult to feel under the prevailing conditions, and attempts to count its frequency were given up. The work of Donald, Bishop & Wade (1955) suggests that intense peripheral vasoconstriction may have been present, which would account for the difficulty in feeling the pulse.

Respiratory rates

Respiratory rates were not measured in these experiments, but numerous observations of these were made on other occasions. During normal climbing the respiratory rate was found to be a multiple, or submultiple, of the number of steps per minute, and one changed abruptly from one rate to another. Rates of 35–50 respirations/min were recorded at altitudes between 18,000 and 21,000 ft. in normal climbing, with rates of 55–60 during short bursts of maximal activity. Respiratory rates as high as 50/min could be kept up for long periods. In the step tests the respiratory rate was always the same as the stepping rate, except in the maximum work tests at 20,500 and 21,200 ft., where several respirations per step were taken, though the details were not recorded.

Maximum ventilation during exercise and maximum voluntary ventilation at rest

The maximum ventilation during exercise (M.E.V.) and the maximum voluntary ventilation (M.V.V.) during rest both increased by about 20% at 20,500–21,200 ft. compared with the values at low altitude (6000–7000 ft.) (Table 7).

TABLE 7. Maximum exercise ventilation and maximum voluntary ventilation (mean of three readings)

Subject	*A* Maximum exercise ventilation (l./min) BTPS	*B* Maximum voluntary ventilation (l./min) BTPS	$\frac{\text{M.E.V.}}{\text{M.V.V.}}$
	Below 6000 ft.		
G.P.	119·4	174·1	0·69
M.P.W.	107·3	168·0	0·64
C.W.	106·8	159·1	0·67
(*a*) Mean	111·2	167·1	0·67
	20,500–21,200 ft.		
G.P.	137·1	221·6	0·62
T.S.	164·5	—	—
	231·5	—	—
M.P.W.	130·8	202·6	0·65
C.W.	—	173·0	—
(*b*) Mean	134·0*	199·1	0·67
(*c*) $\frac{(b)\times 100}{(a)}$	120·5	119·1	—

* Values on T.S. excluded.

The ratio of maximum exercise ventilation to maximum voluntary ventilation was between 0·6 and 0·7 and did not change significantly with altitude. Two experiments, however (one on E.P.H. at 13,200 ft. and the other on T.S. at 21,200 ft.), showed that very high rates of ventilation, probably approaching the maximum voluntary ventilation, may occasionally be observed in short periods of maximum exercise just before the breaking point.

Cotes (1954) demonstrated a 25% increase in maximum voluntary ventilation in subjects exposed to a similar reduction in barometric pressure in a chamber at sea level, and showed that it could be accounted for by the reduced work of breathing air of low density. Although this offers a sufficient explanation of the present findings, some evidence for work hypertrophy of the respiratory muscles was also obtained. In one of the subjects studied (G.P.) additional observations were made at the end of the expedition when the party was at Katmandu (4000 ft.) and a M.V.V. of 210 l./min (mean of three tests) was recorded, whereas at the beginning of the expedition the M.V.V. had been 170 l./min. and at no time subsequently has it exceeded this value.

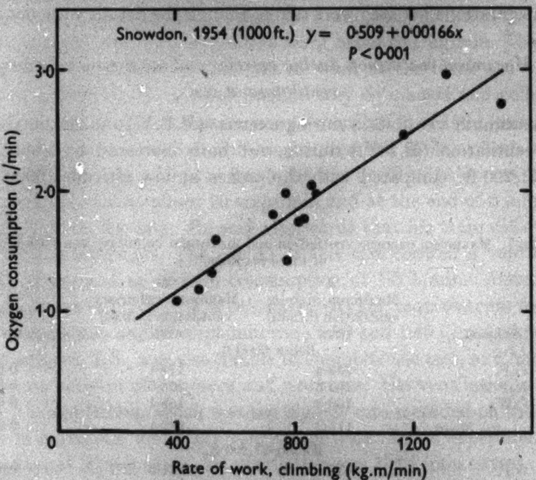

Fig. 5. Oxygen consumption and rate of work, climbing, in men walking uphill at various controlled rates near sea level (Mount Snowdon).

Climbing at controlled rates at low altitude

Fig. 5 shows the relation of oxygen consumption to climbing work in men walking up a hill near Mount Snowdon. Each plotted point represents a single measurement on one of the four subjects, and a linear regression has been fitted to the data. These results, on men climbing at controlled rates, show more scatter than the data on Himalayan climbers walking uphill at their natural pace. It can also be seen that the slope of the graph is steeper than the corresponding graph representing climbing performance in the Himalayas, the explanation being that climbing at low work rates in the Himalayas was less efficient because of the more difficult ground at altitude.

Climbing efficiency

In calculating the efficiency of walking uphill the accepted practice since the time of Zuntz has been to define the rate of work as the product of the total weight raised and the rate of vertical ascent. Gross efficiency is defined as the thermal equivalent of the rate of work divided by the thermal equivalent of the total oxygen consumption, and net efficiency as the thermal equivalent of the rate of work divided by the thermal equivalent of the total oxygen consumption minus the oxygen consumption of metabolic functions not connected with climbing. The value to be assigned to this 'non-lifting' metabolism has been variously calculated. Zuntz *et al.* (1906) and more recently Erickson, Simonson, Taylor, Alexander & Keys (1945–46) deducted the metabolic cost of horizontal walking at a speed equivalent to the horizontal component of climbing. Smith (1922) made a correction for step-lifting. Others have deducted the basal metabolism or the metabolism of standing. Orsini & Passmore (1951) have suggested that the oxygen consumption of walking downhill, instead of horizontal walking, should be deducted.

The present method makes use of the graphs of oxygen consumption and rate of work shown in Figs. 3 and 5. The thermal equivalents of oxygen and mechanical work have been taken as being 4·9 kcal/l. O_2 and 0·0024 kcal/kg.m respectively. The 'non-lifting' oxygen consumption has been taken as equivalent to the oxygen consumption extrapolated to zero rate of work. On this assumption the slope of the graph of oxygen consumption and rate of work is inversely proportional to net efficiency, and in cases where the graph is a straight line net efficiency is constant.

In the step tests and climbing at low altitude the oxygen consumption at zero rate of work was respectively 0·4 and 0·5 l./min, values which can be regarded as intermediate between the oxygen consumption of standing and slow horizontal walking. Net efficiency was 28% for walking uphill, which is comparable with Durnin's (1955) results, and 17% for step tests. The low value of net efficiency in step tests is accounted for by the fact that the negative work of stepping down is not taken into account. If one regards the difference in energy expenditure between walking uphill and stepping as a measure of this negative work, the energy expenditure of stepping down comes out at about 50% of the energy expenditure of stepping up, which is comparable with Zuntz's early results on men walking downhill (Zuntz *et al.* 1906).

According to the present method of calculation, the net energy expenditure of stepping and of climbing a hill at low altitude was constant over the range of rates of work considered. If a lower value for non-lifting oxygen consumption, that is, basal oxygen consumption, were used, the values for net efficiency would fall off at low rates of work.

In Fig. 6 comparison is made between the gross efficiency of climbing a given hill in Wales and of climbing under varying conditions in the Himalayas. Also shown is the net efficiency graph for climbing in Wales. For reasons given below, the net efficiency of the Himalayan climbers cannot be calculated by this method.

The Welsh results indicate that whereas net efficiency was constant, gross efficiency fell off increasingly at low rates of work. The reason for this is that at low rates of work the 'non-lifting' oxygen consumption becomes an increasingly large fraction of the total oxygen consumption.

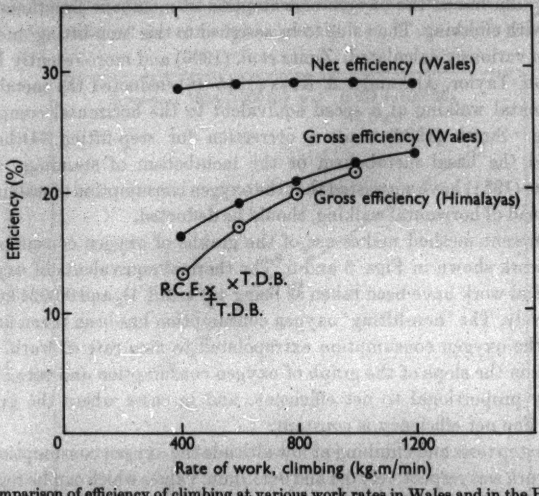

Fig. 6. Comparison of efficiency of climbing at various work rates in Wales and in the Himalayas. +, gross efficiency 20,000 ft., soft snow, no extra oxygen; ×, gross efficiency 26,000–27,000 ft. on closed circuit oxygen.

The curve for climbing in the Himalayas shows the same effect to a greater degree. In this case, low rates of work were associated with high altitude and deterioration of ground. The difference between the curves can be attributed mainly to the effect of ground rather than of altitude, since standard exercise studies have shown that altitude alone has no measurable influence on efficiency. The effect of ground in reducing efficiency at low rates also explains the more gentle slope of the graph of oxygen consumption: rate of work for mountain climbing as compared with climbing on a given track, and the obviously false value for 'non-lifting' oxygen consumption given by extrapolating to zero.

The crosses shown in Fig. 6 represent individual results for climbing under extreme conditions. The values for T.D.B. and R.C.E., climbing between

26,000 and 27,000 ft., have been calculated from information supplied by T.D.B. about the loads carried, the rate of climb, and the amount of oxygen delivered by their closed-circuit oxygen apparatus. The two men, roped together, climbed steadily at 900 ft./hr, kicking steps up a steep snow slope. The oxygen consumption, as calculated from the pressure changes in the oxygen cylinders, was 2·25 and 2·0 l./min respectively. This gives a gross efficiency of only 11–12% compared with 18% for the same rate of work under easy conditions near sea level.

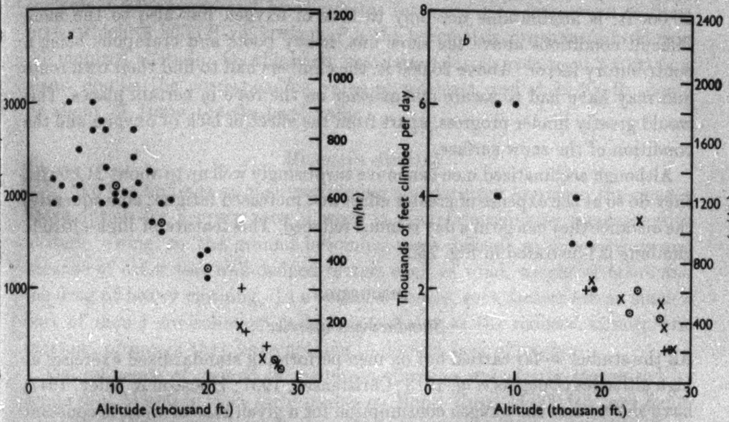

Fig. 7. Climbing rates (*a*) and height climbed in a day (*b*) on the British Himalayan expeditions of 1952 and 1953 and on previous Everest expeditions. Heights plotted are the means of initial and final heights. ●, Mount Cho Oyu (1952) and Mount Everest (1953); ⊙, Mount Everest (1924), Norton; +, Mount Everest (1933), Shipton and Smythe; ×, Mount Everest (1952), Lambert.

Climbing performance

The relation between climbing performance and altitude is illustrated by Fig. 7, in which records of climbing rates and total height climbed in a day have been plotted against mean altitude, i.e. the mean of the initial and final altitudes. The values given for altitudes up to 22,000 ft. have been taken from notes made by the author on the Mount Cho Oyu Expedition and the 1953 Mount Everest Expedition. They refer to men walking uphill under typical conditions, carrying loads of less than 25 lb. Ascents on very gentle slopes like glacier névés, where the horizontal distance covered is more significant than the height, have not been included. The values referring to altitudes above 22,000 ft. have been taken from published accounts of previous Everest expeditions. No relevant data are available from the 1953 Everest Expedition because oxygen equipment was used on the two assaults and, in the preparatory phase, Europeans climbing above 22,000 ft. without oxygen apparatus were either working on the route or escorting parties of heavily laden Sherpas. Fig. 7*a* illustrates the wide variation in rate of climbing at intermediate altitudes, and the steep decline in performance above 20,000 ft. The rates of 3000 ft./hr at altitudes below 12,000 ft. were observed only on the return march when the climbers were underweight but very fit. Contributing to the high rates of climbing at these altitudes was the fact that the climbers wore only 'gym' shoes and shorts. The decline in performance above 20,000 ft. is attributable not only to lack of oxygen but also to the more difficult conditions above the snow line, heavy boots and crampons being a contributory factor. Above 26,000 ft. the climbers had to find their own route and may have had to secure one another on the rope in certain places. This would greatly hinder progress, apart from the effect of lack of oxygen and the condition of the snow surface.

Although acclimatized men can move surprisingly well up to about 21,000 ft., they do so at the expense of greater effort and increased fatigue; consequently, the distance they can go in a day is much reduced. This feature of high-altitude climbing is illustrated in Fig. 7*b*.

DISCUSSION

Standardized exercise

All the studies so far carried out on men performing standardized exercises at high altitudes (Douglas *et al.* 1913; Christensen, 1937; Houston & Riley, 1947) have shown that the oxygen consumption for a given rate of work is constant and independent of altitude. The extra oxygen consumption of increased cardiac and respiratory work at high altitude is apparently too small to be measurable, at least under field conditions. This observation is in line with the more recent work as reviewed by Shephard (1955) on the extra oxygen consumption associated with hyperventilation induced by various methods at sea level, and with the finding of Christensen & Forbes (1937) that in the acclimatized subject the cardiac output for a given intensity of work at altitude is, in fact, the same as it would be for a similar intensity of work at sea level. The subject of the metabolic cost of ventilation has been reviewed in detail by Shephard (1955) who regards the generally accepted figure of 1·20 ml. O_2/l. as being probably correct. On this basis, the extra oxygen consumption of increased ventilation due to altitude would hardly exceed 75 ml./min, even in extreme circumstances and leaving out of account the savings in respiratory work due to reduced air density.

The second principle illustrated by the present results is the finding of Christensen (1937) that ventilation (STPD) is virtually independent of altitude in the acclimatized subject and bears a nearly constant relation to oxygen consumption over a wide range of rates of work. This finding has several important implications. As pointed out elsewhere (Pugh, 1957) in connexion with resting ventilation where the same rule applies, it means that the respiratory adaptation to altitude is such that the reduced air density at altitude and the increased proportion of the lung space occupied by water vapour (the vapour pressure of water in the lungs being constant) are almost completely compensated. If the oxygen consumption for a given activity at sea level is known, the corresponding ventilation (BTPS) at any given altitude can be predicted simply by multiplying the oxygen consumption by the ventilation equivalent for oxygen and by the factor for converting litres STPD to litres BTPS, which is $863/(P-47)$ where P is the barometric pressure (mm Hg) at the required altitude. On this basis, ventilation during exercise at 20,000 ft. is about double the corresponding value at sea level, and at 29,000 ft. should be about $3\frac{1}{2}$ times the sea-level value.

Mountain climbing

Unlike the findings on men performing standardized exercises, the oxygen consumption for a given rate of work in mountain climbing increases with altitude owing to the ground becoming more difficult as one ascends and because of other less well-defined factors such as wind, weight of boots and the drag of heavy clothing. In a sense, of course, such factors are as much a part of man's environment in high mountains as the reduced atmospheric pressure, although they have nothing to do with lack of oxygen.

The influence of ground in mountain climbing was clearly recognized by Zuntz *et al.* (1906) in their early studies on Monte Rosa (14,600 ft.), and Zuntz pointed out the extreme difficulty of predicting oxygen consumption from rates of climbing, particularly for men climbing on snow and ice. However, on plotting the available data on men climbing at their habitual pace under easy conditions, it was found that a linear relation existed between oxygen consumption and rate of work, which has nearly the same slope both for the Alpine and Himalayan conditions. This empirical relation provides a useful, if only approximate, basis for calculating oxygen consumption from rates of climbing for the conditions specified, to which corrections might be applied for more difficult conditions.

The observations made on men climbing at their habitual pace at 15,500 and 20,000 ft. which were made on the expedition to Mount Cho Oyu in 1952 were of considerable value in planning the oxygen equipment for the successful expedition to Mount Everest the following year. The range of altitude, from 15,000 to 20,000 ft., corresponded with the range of 'effective' altitudes within which the climbers on the higher slopes of Everest would be working when using their oxygen apparatus. The high levels of oxygen consumption and ventilation observed focused attention on the need for adequate supplies of oxygen and breathing apparatus designed to keep respiratory pressures within acceptable limits at high rates of ventilation. The rates of climbing recorded confirmed the opinion held by experienced Himalayan mountaineers that, after suitable acclimatization, their performance at 20,000 ft. in the Himalayas is much the same as it is at 12,000–14,000 ft. in the Alps (where ascents are usually too rapid to allow time for acclimatization). Further support for this view is provided by film sequences taken on Mount Everest of parties climbing on the Khumbu Glacier, which showed no obvious slowness or weakness of movement in the climbers below 20,000 ft. These rates of climbing were considered by the climbers themselves to be normal for the conditions, and were consistent with observations made on other occasions (Fig. 7). However, such rates could not be kept up for as long as at lower altitudes, and much more effort was involved. Subjectively one was aware all the time of breathing near the maximum, and of heaviness and weakness of the legs. Any increase in gradient, or deterioration in snow conditions, would bring one to a halt to recover breath. In view of these subjective effects, it was not surprising to find evidence that climbers at 20,000 ft. were working very near to capacity with regard to oxygen consumption and ventilation. This is a finding which could hardly have been predicted from studies of controlled exercise.

Other points illustrated by these experiments are: (1) the close connexion of oxygen consumption with the feeling of effort in men climbing at their habitual pace; to what extent this depends on regular training and practice is an open question, but certainly the results were much more uniform than those obtained in Wales on men climbing at various controlled rates and not at their natural pace: and (2) the wide variation of oxygen consumption and ventilation in large and small men; this was a matter of some practical importance in connexion with oxygen equipment. With open-circuit apparatus where the concentration of oxygen in the inspired gas mixture depends on the ventilation rate, the large man is at a twofold disadvantage, for not only is his oxygen requirement higher but the concentration of oxygen he inhales is lower than would be the case for a small man.

Exhausting exercise of short duration

The values for maximum oxygen consumption observed in members of the Mount Everest team at sea level were lower than might have been expected in a highly selected group of mountaineers. Although this is partly explained by their poor state of training at the time of the experiments, the results are so inferior to published reports on first-class middle- and long-distance runners as to raise the presumption that mountain climbing, being a non-competitive sport, does not incite climbers to undergo the intensive training which would develop the extreme aerobic capacity found in some track athletes. Some improvement in the values obtained at sea level on members of the Everest party would be expected, however, had the subjects been as fit as they were on the expedition itself, and this should be taken into account in comparing the results at sea level with the results at high altitude and with the data of other workers. Judging by differences in rates of climbing at the beginning and at the end of Himalayan expeditions, such improvement would amount to at least 10%, and this would be consistent with experimental studies (Robinson & Harman, 1941*a*, *b*).

The use of a step test in determining maximum oxygen consumption at 20,000 ft. is open to the criticism that the step test is a form of exercise in which the work load is unevenly distributed between the two legs, and therefore the maximum oxygen consumption may be underestimated. The observations on one of the subjects (G.P.) at sea level showed a difference of only 0·3 l./min in maximum oxygen consumption between running and exhausting step tests; the corresponding difference at 20,000 ft. would be about half as great.

Evaluation of the effect of altitude on maximum work ventilation is complicated by the fact that the period of work at altitude was 4–6 min compared with 10 min at sea level. Whereas there is no reason to suppose that this would affect the maximum oxygen consumption, it may have an important influence on ventilation, since, as already pointed out, studies at sea level have shown that some subjects in the shorter exercise may exhibit excessive respiratory stimulation with ventilation equivalents up to 30 l./l. O_2 (Åstrand, 1952*a*). This type of response was not seen in the sea-level experiments on the Everest party whose period of work was 10 min, but did occur at altitude in experiments where the period of work was 4–6 min. The effect cannot therefore be regarded purely as an effect of altitude, although in both cases it is a partial response to lack of oxygen (Pugh, 1954; Åstrand, 1954).

Comparison of maximum oxygen consumption with oxygen consumption during normal climbing

The chart shown in Fig. 8 has been constructed from the experimental data on Mount Everest and Mount Cho Oyu, as well as derived values calculated with the aid of the oxygen-consumption–work-rate diagram (Fig. 3) from records of rate of climbing and body weight. The following example serves to illustrate this procedure. Shipton and Smythe (Shipton, 1943), on the 1933 Mount Everest Expedition, climbed from 23,000 to 24,500 ft. in $1\frac{1}{2}$ hr, which is equivalent to a climbing rate of 1000 ft./hr or 5·08 m/min. Shipton's weight on the Mount Cho Oyu Expedition (1952) was 63·6 kg, and since he is of lean build it is safe to assume that his weight has not altered greatly in the intervening years. Adding 7·7 kg for the weight of clothing and equipment, the total weight raised is 71·3 kg, and the work rate $71{\cdot}3 \times 5{\cdot}08 = 362$ kg.m/min. From Fig. 7 the oxygen consumption corresponding to this work rate is 1·4 l./min. Similar values have been calculated for climbs above 27,000 ft. by Shipton and Smythe in 1933 and Lambert and Tensing in 1953 on which

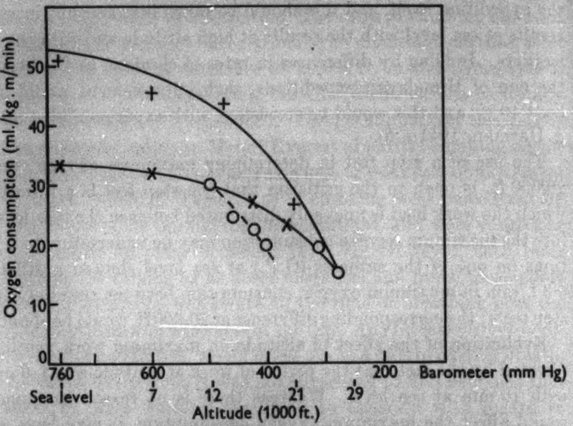

Fig. 8. Comparison of maximum oxygen consumption and oxygen consumption during normal climbing at various altitudes. Mean experimental values are represented by: +, maximum oxygen consumption; ×, oxygen consumption in normal climbing; ○, calculated values for normal climbing based on records of rate of climbing and body weight (see text).

oxygen apparatus was not used. The values for altitudes above 27,000 ft., however, include the time spent resting, since sustained climbing is not possible at such heights. Also shown in Fig. 8 are values for partially acclimatized men which have been calculated from records of an ascent to 18,000 ft. 4 days after the arrival of the 1953 Expedition at its main base at 13,000 ft.

The chart illustrates the decline in maximum oxygen consumption from low altitude upwards in contrast with the oxygen consumption of normal climbing, which in well acclimatized men shows but little impairment below 18,000 ft. Partially acclimatized persons, on the other hand, do show considerable impairment at this altitude. It can be seen clearly that the relatively high oxygen consumption in normal climbing is achieved by working progressively nearer to capacity. Eventually a point is reached at around 27,000 ft. where sustained progress is no longer possible and the climber has to keep stopping to recover his breath. Norton (1925) described climbing at 28,000 ft. without oxygen equipment as follows—'My ambition was to do 20 consecutive paces uphill without a pause, to rest and pant, elbows on bent knees, but I never remember achieving it—thirteen was near the mark. Every 5 or 10 minutes we had to sit down for a minute or two, and we must have looked a sorry couple.' Somervell, who was Norton's companion on this occasion, described taking 7–10 complete respirations for every single step forward and upward (Somervell, 1925). On a Swiss expedition to Mount Everest in 1952, Lambert (1953) reported having to rest every 20 paces while climbing above 27,000 ft.

Assuming a respiratory rate of 50 respirations/min, Norton and Somervell must have rested about every 2 min. In such a way, by alternating short periods of rest and work, climbers at great altitudes have been able to carry on for many hours without becoming totally exhausted. A similar device is used by Himalayan porters carrying heavy loads at low altitudes. On the expedition to Mount Cho Oyu observations were made on a group of 25 porters carrying loads of up to 100–140 lb. (45–64 kg) at altitudes between 2500 ft and 7000 ft. The weight and height ranges of 11 of these men was 95–125 lb. (43–57 kg) and 58–63 in. (147–160 cm) respectively. On steep hillsides they climbed continuously for periods varying from 2–3 min at a time, resting for ½–1 min between each bout of climbing. In this fashion ascents of 3000 ft. and more were accomplished in the course of a 12 hr working day without apparent fatigue. Experimental studies of intermittent exercise of this type at sea level have been reported by Christensen (1956). He found that exercise at an intensity causing complete exhaustion in 9 min (oxygen consumption 4·6 l./min) could be kept up for an hour or more without fatigue by alternating half-minute periods of work and rest.

Climbing efficiency

The application of the concept of efficiency to mountain climbing has uses and abuses. Hill (1927) calculated that the climbing rate of a 75 kg man at an oxygen consumption of 2·0 l./min would be 2380 ft./hr under good conditions, assuming a gross efficiency of 20%. These values were very close to those actually observed at low altitudes in the Himalayas. Roxburgh (1947) predicted that the oxygen consumption of climbers on Mount Everest using oxygen apparatus would be 1·9 l./min, which is close to the rates observed by T.D.B. using the closed-circuit equipment between 26,000 and 27,000 ft. This calculation was, however, based on an assumed climbing rate of 1600 ft./hr and an efficiency of 20%, whereas in the event only 900 ft./hr and an efficiency of 11–12% were attained. (In calculating efficiency, resting metabolism was deducted from the total metabolism; the corresponding value for gross efficiency would be 17%.)

It is generally accepted that the net efficiency of climbing is greatest in men walking rather fast on gentle slopes. Thus Erickson *et al.* (1945–46), in their treadmill studies, found maximum efficiencies of 35–40% with the treadmill set at a gradient of 1 in 20. Mountaineers, however, prefer slower speeds and steeper gradients than this, and calculation shows that their method is, in fact, more economical as regards total energy expenditure. For example, in one of the experiments on the approach to Mount Everest, M.P.W. climbed 2100 ft. in 1 hr on a gradient of approximately 1 in 5, his horizontal speed being of the order of 2·0 miles (3·2 km)/hr. His oxygen consumption was 1·9 l./min, which gives a total calorie cost of 576 kcal. If he had made the same ascent on a

1 in 20 gradient at 3·5 miles (5·6 km)/hr, with a net efficiency of 40%, his energy expenditure would have been 1199 kcal.

According to the present results, the over-all efficiency of climbing at high altitude is but little over half the efficiency of climbing at sea level, even under easy conditions. This is partly due to the ground and associated factors, and partly to the increase in the ratio of 'non-climbing' to 'climbing' metabolism at low rates of work. In preparing oxygen equipment for climbing Mount Everest calculation of oxygen requirements and rates of climbing based on a gross efficiency of 20% would have led to a grossly inadequate supply of oxygen being taken. This mistake was made on previous expeditions to Mount Everest, and led to arguments between physiologists and engineers at the planning stage of the 1953 Everest Expedition over the amount of oxygen to be sent out with the Expedition and the rates of climbing to be expected from its use.

Altitude limit for climbing without oxygen apparatus

The question whether it is physically possible for men to climb to the summit of Mount Everest without using oxygen equipment remains unanswered. Since the first full-scale expedition in 1922, no less than nine men have climbed by their own unaided efforts to about 28,100 ft. and returned alive. The difference in atmospheric oxygen pressure between this height and the summit is only a matter of 10 mm Hg, which is within the range of variation due to weather conditions. Extrapolation from the curves in Fig. 8 suggests that the maximum oxygen consumption of an 80 kg man at the summit of Mount Everest would be of the order of $12 \times 80 = 960$ ml./min, which would suffice for walking on the level and perhaps for climbing a gentle slope under easy conditions. This calculation is supported by the statements of climbers on the 1953 expedition and by the two Swiss parties who reached the summit in 1956, all of whom reported being able to walk on the level ground at the summit of Mount Everest without wearing their oxygen equipment.

Three other factors, however, make it unlikely that the mountain could be climbed without oxygen equipment without serious risk. The first is the greatly reduced physical endurance at these heights; the second is the length of time required to cover the last 1000–1500 ft.; it might prove impossible to complete the ascent and get down to shelter before nightfall; and the third factor is the effect of lack of oxygen on the brain, which is known to be exaggerated by exercise. The two Swiss parties who reached the summit in 1956 remained there for 2 hr without using their oxygen equipment. E.P.H., on the other hand, in 1953, found he had to put on his set again after 10 min without it on account of incipient mental confusion and clumsiness. Although both British and Swiss parties found they could walk about the level fairly easily without extra oxygen, none claimed they could have reached the summit without it.

SUMMARY AND CONCLUSIONS

1. In step tests at various altitudes up to 20,500 ft. and at intensities of work that could be maintained without strain, oxygen consumption and ventilation (STPD) were proportional to the rate of work and virtually independent of altitude.

2. In men climbing at their habitual pace at altitudes up to 20,000 ft. strikingly reproducible results were obtained on successive ascents over the same ground, without any control of the rate of work other than the subjective feeling of effort. Ventilation volumes of 72–124 l./min (BTPS) and oxygen consumptions of 1·4–2·2 l./min were observed at 20,000 ft., the variation being mainly accounted for by body size. In contrast with standardized work, as in step tests, the efficiency of climbing decreased with altitude.

3. Evidence is put forward that the relatively high performance of climbing at great heights was achieved by working near to capacity. At about 27,000 ft. steady climbing was no longer possible and the climber had to rest every few steps.

4. The maximum oxygen consumption of members of the Everest party at sea level was 3·6–3·9 l./min which was low in comparison with values recorded in track athletes. These low values were partly accounted for by lack of training at the time when the experiments were carried out. At 20,500 ft. maximum oxygen consumption was approximately half the maximum value at sea level.

5. Maximum ventilation during exercise and maximum voluntary ventilation at rest increased by 20% at approximately 21,000 ft. This was attributable to reduced atmospheric density.

6. The ratio of maximum ventilation during exercise to maximum voluntary ventilation at rest did not change significantly at altitude except in some subjects working to exhaustion in 2–4 min, whose ventilation rose to very high values probably approaching maximum voluntary ventilation just before the breaking point. This was not an effect of altitude *per se*, since similar findings have been reported at sea level.

7. Records of performance in mountaineers ascending from low altitudes to 28,000 ft. are presented. The effect of altitude and terrain on climbing efficiency and the question of whether it is possible to ascend without oxygen equipment to the summit of Mount Everest are discussed.

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THE INFLUENCE OF AGE ON PERIPHERAL VASODILATATION IN A HOT EVIRONMENT

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The effect of age on some of the reactions of men to a hot environment has recently been described (Hellon, Lind & Weiner, 1956). Men aged about 45 years were shown to differ in their reactions from men aged 20 in two ways: (1) the older subjects showed a greater degree of forearm vasodilatation, and (2) their sweat glands did not respond so rapidly to the increased heat load of exercise. Further observations on the responses of the sweat glands have already been published (Hellon & Lind, 1956). The purpose of the present investigation has been to examine in more detail the effect of age on the forearm circulation under hot conditions.

The experiments of Hellon *et al.* (1956) included four periods of exercise in the hot room routine and it was not possible to ascertain how far work *per se* might have been responsible for the greater dilatation in the forearms of the older men. The present experiments fall into three series. The first deals with subjects of different ages exposed to heat while resting; the second series includes one work period in the routine; finally the same work is performed under comfortable conditions.

METHODS

The subjects in these experiments comprised two age groups. The younger group consisted of undergraduate students aged between 17 and 26 years, with a mean of 20 years. For the older group, college servants were selected and their average age was 47·5 years (range 41–57). The subjects, who were dealt with in pairs of one young and one older man, were medically examined on arrival at the laboratory and considered to be healthy. They rested for 60 min in a room where the temperature on any one occasion was between 24 and 27·5° C. The exposure to the controlled environment lasting 150 min then followed and in all experiments the first 65 min were spent sitting. Where work was performed, this commenced 65 min after entry to the climatic chamber and continued for 20 min; the remaining 65 min of exposure were spent sitting. The work consisted of mounting and dismounting from a stool 30·5 cm high at the rate of 12 steps/min in time to a signal bell.

In the experiments of the first and second series the climatic chamber was maintained at

38·0° C dry-bulb temperature and 30·5° C wet-bulb temperature. When work under comfortable conditions was studied in the third series, the corresponding temperatures were 26° and 18° C. In all experiments air movement was about 20 cm/sec. While in the climatic chamber the subjects wore only an athletic supporter.

Blood flow was measured in the right forearm with a mercury-in-rubber strain gauge plethysmograph (Whitney, 1953), placed round the forearm midway between elbow and wrist. This instrument has been shown to give similar readings of blood flow to a conventional venous-occlusion plethysmograph (Clarke & Hellon, 1957). The arm was supported at heart level on a special rest attached to the subject's chair. On each occasion that blood flow was measured, an arterial occlusion cuff (200 mm Hg) was inflated at the wrist and, after a pause of 1 min, four inflow records were taken by applying pressures of 60 mm Hg to an inflatable cuff round the upper arm. Pulse rates were counted from the plethysmograms. Blood pressures were measured on the left arm by auscultation before entry to the climatic chamber and 40, 100 and 140 min after entry.

Rectal temperatures were recorded at 5 min intervals throughout the experiments from a thermocouple inserted 8 cm beyond the internal anal sphincter (Lind, Hellon, Weiner & Jones, 1955). Skin temperatures were measured at eleven standard points on the body using a roving thermocouple (30 s.w.g.).

RESULTS

1. *Subjects sitting in heat*

The observations were made on six pairs of young and older subjects. Fig. 1 shows forearm blood flows in eleven of the twelve subjects before and during exposure to heat. Readings were not obtained on one subject owing to a fault in the recording camera.

Blood flow before exposure to heat tended to be higher in the older men, but the room temperature varied from 24 to 27·5° C and these results could not be regarded as strictly comparable. During the first 70 min of exposure to heat, there was a considerable scatter of results with no clear distinction between the means of the two age groups. At 90 min after entering the hot room, however, there was a difference, the blood flows of the older men having risen to a mean level of 11·6 ml./100 ml. forearm/min and those of the younger group having declined to a mean value of 8·5 ml./100 ml./min. This difference remained about the same for the remainder of the exposure to heat.

The mean values for the younger men were clearly much affected by one of the students who had extremely high blood flows. These were more than twice those of other students and higher than those of any of the older men. It is considered that he was exceptional in this respect, particularly when the blood flows found in the twelve students described in the next section of this paper are noted. The measurements of skin and rectal temperature in this student were within the same range as the rest of the younger group.

On exposure to heat, the pulse rates in both groups increased by 15–20 beats per min above their pre-heat values and on no occasion did the group mean values differ significantly during the experiment. The mean values for blood pressure in the two age groups are shown in Table 1. The older subjects had significantly higher systolic and diastolic pressures, both before entering the hot chamber

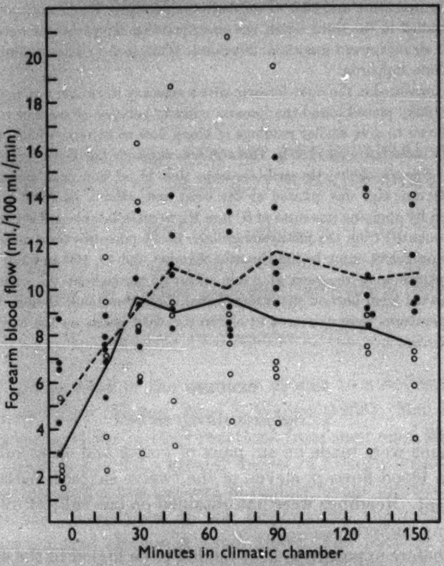

Fig. 1. Forearm blood flows in five young (○) and six older (●) subjects during heat exposure. Each point is the average of four inflow curves. The solid and broken lines indicate the mean results of the young and older groups respectively.

TABLE 1. Mean blood pressures (mm Hg) in the two age groups. Y = young group (6 subjects), O = older group (6 subjects)

	Systolic pressure		Diastolic pressure		Pulse pressure	
	Y	O	Y	O	Y	O
Before heat exposure	117	140	85	99	32	39
After 40 min	110	131	77	102	33	29
After 110 min	105	126	77	99	28	28
After 145 min	110	123	79	92	31	31

and during exposure to heat. The mean values in both age groups tended to fall on exposure to heat. Pulse pressures were very similar in the two groups and did not differ significantly.

Both age groups started with almost identical mean skin and rectal temperatures (see Fig. 2). The changes produced on exposure to heat were similar in each group, but the older men assumed a slightly higher level. At no time were the differences between the groups significant. The rise of skin temperature to a peak at 45 min and the subsequent fall to a steady level at 90 min were presumably connected with the onset of sweating.

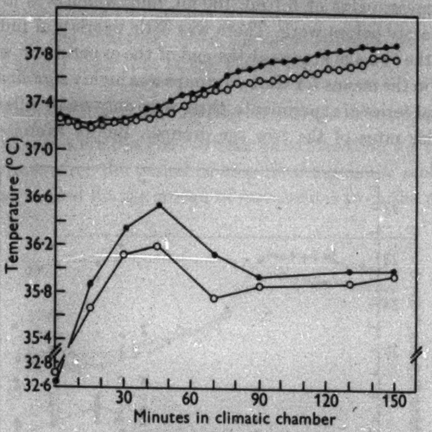

Fig. 2. Mean skin and rectal temperatures in 6 young (○) and 6 older (●) subjects during heat exposure.

2. *Subjects sitting and working in heat*

These experiments were designed to examine the effects of work on forearm blood flow in the two age groups during exposure to heat. Twelve pairs of young and older men were examined and none of these had taken part in the previous series.

Fig. 3 shows the individual forearm blood flow measurements during the experiment. On exposure to heat, the increases in blood flow were comparable to the majority of those found in the previous series. After 45 min exposure to heat, the means of forearm blood flow were 7·0 and 10·9 ml./100 ml./min for the younger and older groups respectively. The corresponding values in the previous series were 6·7 and 10·8 ml./100 ml./min if the exceptional young subject is excluded.

Readings taken 10 min after the work period showed that all the subjects' forearm blood flows had increased. The mean blood flow for the students rose by 3·2 ml. to 10·2 ml./100 ml./min. This level is less than the mean value recorded in the college servants before they started to work. The mean flow of the college servants rose by 5·6 ml. to 16·5 ml./100 ml./min. The difference between the means of the two groups after 95 min exposure is highly significant ($P<0{\cdot}01$). For the remainder of the exposure to heat, the blood flows in both groups declined at about the same rate. At the end of the experiment, 65 min after the end of work, the mean blood flow of the older men was 12·0 ml./100 ml./min, still higher than their pre-work level, but the younger group had a mean value of 6·1 ml./100 ml./min, which was lower than their mean immediately before work. There was little overlap of individual results in men from the two age groups at the end of the experiment, and at this time the difference in the means for the two groups was highly significant ($P < 0{\cdot}001$).

As in the first series of experiments, there were only small differences between the mean pulse rates of the two age groups. Before exercise, the younger subjects had slightly higher mean pulse rates, but following work, this difference was reversed. Individual results showed wide variation and differences between the means were not significant.

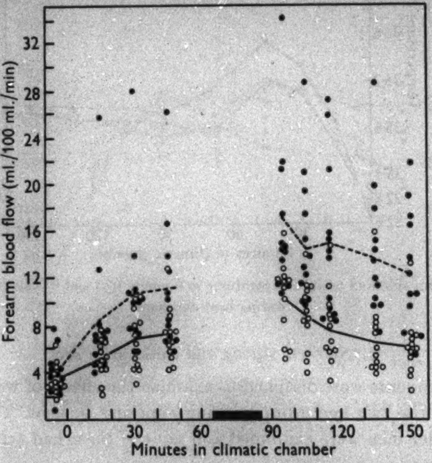

Fig. 3. Forearm blood flows in 12 young (○) and 12 older (●) subjects during heat exposure. The solid and broken lines indicate the mean results of the young and older groups respectively. The black rectangle indicates the duration of the period of work.

The systolic pressure in both age groups decreased by about 10 mm Hg on exposure to heat. Diastolic pressure was not appreciably altered. The older men showed higher systolic and diastolic pressures than the younger men before and during the experiment, but because of the wide individual variation their results did not differ significantly from those of the younger men. The means of the pulse pressures for the two age groups were almost identical.

The mean values for skin and rectal temperatures are plotted in Fig. 4. There was no significant difference between the mean skin temperatures until the readings taken after the work period, 95 min after entry to the chamber. At this time the older men had higher skin temperatures ($P < 0{\cdot}001$); at the end of the experiment, 65 min later, the difference was still just significant ($P < 0{\cdot}05$).

Rectal temperatures in the two groups were significantly different only at 115 min after entry to the chamber, when the older subjects had a mean temperature 0·2° C higher than that of the young men.

3. *Subjects sitting and working in a comfortable climate*

These experiments were designed to seek an explanation for the increase in forearm blood flow after the period of step-climbing in the second series. If the increase was caused by the spread of metabolites from the muscles of the legs, then a similar increase would be expected when the same work was performed under cool conditions. On the other hand, if the rise in body temperature was responsible, a cooler environment, in which the change of body temperature would be less, might reduce or even eliminate the increase in blood flow following work. The experimental routine was the same as in the second series, but the dry-bulb temperature was only 26° C.

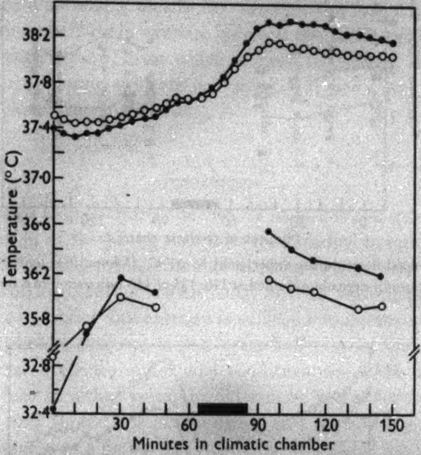

Fig. 4. Mean skin and rectal temperatures in 12 young (○) and 12 older (●) subjects. The black rectangle indicates the duration of the period of work.

The forearm blood flows of the ten pairs of subjects are plotted in Fig. 5. Step-climbing for 20 min produced only a small rise of blood flow. The mean values for both age groups showed increases of less than 1 ml./100 ml./min, compared with increases of 3·2 and 5·6 ml./100 ml./min for the young and older groups respectively when they performed the same work in the heat. In only two of the subjects were blood flows observed after the work which were consistently higher than they had shown before the work. In both these

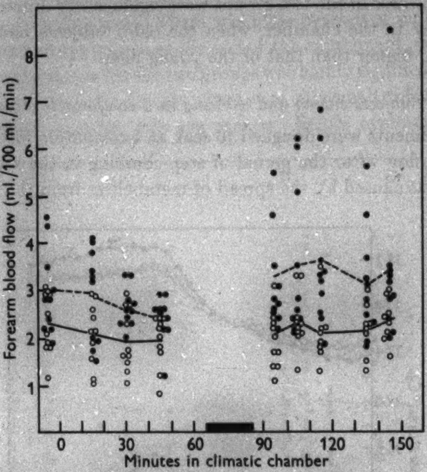

Fig. 5. Forearm blood flows during experiment at 26° C. Conventions as for Fig. 3. Points for two of the young group were not obtained at 115, 135 or 145 min owing to a mechanical failure.

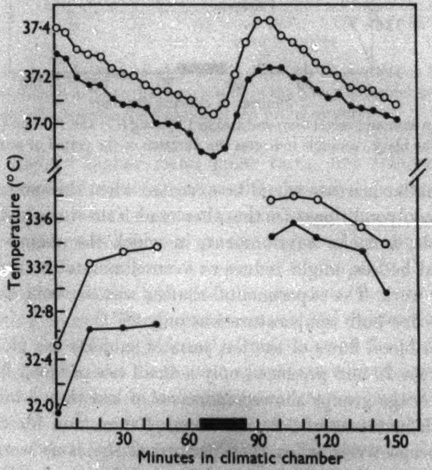

Fig. 6. Mean skin and rectal temperatures during experiment at 26° C. Conventions as for Fig. 4. Note change in ordinate scale.

men blood flow increased gradually from the readings taken 10 min after exercise to those taken 55 min later at the end of the experiment. It is difficult to attribute this slow increase to the effect of work, since in that event, the blood flows would be expected to have shown an immediate rise followed by a gradual fall, as shown in Fig. 3.

Fig. 6 shows the mean values in each group of skin and rectal temperatures throughout the experiment. The changes in the mean results were similar for both groups. The environment was evidently not sufficiently warm to allow sitting men to maintain heat balance while they were unclothed, and the rectal temperature showed a steady fall through the first 65 min. The heat production during the 20 min exercise restored the rectal temperature to its initial level in both groups, after which it again fell slowly. Unlike the two previous series, the young subjects had slightly higher rectal and skin temperature throughout the experiment.

DISCUSSION

The present results help to clarify the various factors which were acting on the forearm circulation of the young and older men studied in our previous work (Hellon *et al.* 1956). The prime stimulus to provoke a greater vasodilatation in the forearms of the older men is evidently not the performance of exercise. Exposure to heat without any exercise is sufficient to induce a forearm blood flow which, in the older subjects, is on average half as great again as that in the younger subjects. Exercise under cool conditions has little or no effect on forearm blood flow in either young or older men, and it follows that similar exercise in the heat should not play a direct part in raising peripheral blood flow in the non-exercising limbs. It is evidently the added heat load resulting from the work which is responsible for the increased blood flow following exercise under hot conditions.

Such an explanation is not necessarily invalidated by the experiments at 26° C, where little rise in forearm blood flow took place, despite the appreciable rise in rectal temperature accompanying the work. Here the subjects were unable to maintain heat balance resting in the first hour and rectal temperature fell steadily. The heat load of the work did no more than restore that temperature to its starting level, with a rise of some 0·4° C in both age groups. Under hot conditions, the rises in rectal temperature during work were 0·48 and 0·62° C in the younger and older groups respectively, but these increases were superimposed on an already elevated temperature due to the warmer environment, so that the rise above the initial level of the experiment approached 1° C.

The older subjects tended to have higher blood pressures both before and during the heat exposure, but the difference in blood flow between the two age groups cannot be attributed to this cause. Both age groups showed a reduction in blood pressure after entering the hot chamber at a time when forearm blood flow was increasing, and it may be concluded that an active vasodilatation was responsible for the increased blood flow.

The preliminary clinical examination which all the subjects received did not exclude the possibility that some degree of peripheral vascular disease might have been present in the older men. However, peripheral vascular diseases of older people lead to a reduction in blood flow through the peripheral vessels; since blood flow has been shown to increase with age, it appears that such diseases, if actually present, were not influencing the responses of the older subjects in any way.

The older group also tended to show higher blood flows in the period before exposure to heat, but, as has already been stated, room temperature was not accurately controlled and the results have not been analysed statistically. The same tendency was also shown where the temperature was maintained at 26° C (Fig. 5). It appears possible that this difference between the age groups under cool conditions represents the initial stage of the greater blood flow observed when the subjects are heated. A separate study of this age difference under cool conditions will appear elsewhere. Results obtained by several techniques (Barcroft, Bock, Hensel & Kitchin, 1955; Roddie, Shepherd & Whelan, 1956; Edholm, Fox & Macpherson, 1956) have demonstrated that indirect heating causes an increase in flow only in the skin vessels of the forearm. There is no reason to suppose that a similar reaction is not taking place in the present subjects of both age groups and hence it is probable that the higher blood flows of the older subjects are accommodated in their forearm skin.

At present there is no evidence to indicate why men over the age of 40 should show this greatly increased vasodilatation in response to heat. Unless it be supposed that their forearm blood vessels are hypersensitive to the local direct action of heat, the additional vasodilatation could be due to an alteration in the level of vasomotor activity or in the supply of some vasodilator hormone. Since the increase in blood flow accompanying body heating is known to be mediated by the sympathetic supply to the forearm vessels (Barcroft, Bonnar & Edholm, 1947), a hormonal factor is unlikely to be involved.

It appears that the major part of the dilator response to body heating is mediated by dilator fibres (Roddie, Shepherd & Whelan, 1957), although these fibres may act indirectly on the skin vessels through the local formation of bradykinin by the sweat glands (Fox & Hilton, 1957). On this basis, it is tentatively suggested that the greater vasodilatation of the older men is due to the increased activity in their dilator fibres in response to external warmth, or to a heightened sensitivity of their skin vessels to a given amount of dilator activity.

Since, in the hot environment used in these experiments, heat loss was entirely by evaporation, the greater skin blood flow in the older subjects would not be expected to have a marked effect on their heat regulation. Indeed, the differences between the age groups in rectal and skin temperatures were very small. Thus the faster skin circulation of the older men is unnecessary for heat regulation and places a needless burden on the general circulation. The additional blood flow must require a greater cardiac output or, if this is unchanged, a compensatory vasoconstriction elsewhere. Our evidence is not sufficient to decide between these two possibilities.

The subjects in our previous work (Hellon *et al.* 1956) were mineworkers, who were known to be extremely fit and capable of heavy manual work (Lind *et al.* 1955). Since the present subjects, who were medical students and college servants, could not be regarded as in this category, and since they gave similar responses to the miners, it appears that the results reported here are probably applicable to the general male population.

SUMMARY

1. The changes in forearm blood flow on exposure to a hot environment have been compared in young men (17–26 years) and older men (41–57 years).

2. While resting in the heat, the older men have forearm blood flows which are 50% greater than those of the young men.

3. When work is performed in the heat, a further increase in blood flow occurs in both age groups, but the increase is greater in the older men.

4. The increase in blood flow following work does not occur if the work is done at a temperature of 26° C.

5. The consequences of and possible reasons for the greater vasodilatation in the older subjects are discussed.

We should like to thank Dr J. S. Weiner for his advice and encouragement, and the subjects for their whole-hearted co-operation. This work formed part of an essay which received a Ciba Foundation Award for Basic Research relevant to the Problems of Ageing, 1957.

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THE INTERACTION OF pH, TONICITY AND ELECTROLYTE CONCENTRATION ON THE MOTILITY OF DOG SPERMATOZOA

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Hydrogen-ion concentration and tonicity are two of the most important factors influencing the survival of mammalian spermatozoa; the effect of electrolyte, in the form of sodium chloride, has however most probably been over-rated by earlier workers.

The first systematic studies of all three factors were made by Emmens (1947, 1948) who observed the motility of rabbit spermatozoa at various pH levels with diluents of different tonicity and chemical composition. The optimal pH for rabbit spermatozoa was found to be about 7, but they were at least partially motile between pH 5 and 10. Both hypotonic and hypertonic diluents were deleterious at all pH levels, but the relative effects of hypo- and hypertonicity varied with pH. Thus in acid or neutral solutions the spermatozoa were more susceptible to hypo- than to hypertonicity, but in alkaline media the situation was reversed. Contrary to what might have been expected from the early literature (see Anderson, 1945), motility was not significantly affected by changes in the proportion of sodium chloride and glucose in buffered diluents of the same tonicity, except above pH 9.

More recently these studies have been extended to ram, bull and human spermatozoa (Blackshaw & Emmens, 1951). Those of ram and bull showed optimal motility at about pH 7 and of human at about pH 8·5; the spermatozoa of all three species were at least partially motile within a pH range of 5–10, and bull spermatozoa were not completely immobilized even at pH 4·4. In this latter species hypertonic solutions were less harmful than hypotonic media at all pH levels, but the relatively slight adverse effect of hypertonicity could be diminished by partial replacement of sodium chloride by glucose at alkaline pH levels.

There appears to be an almost complete lack of information on the effect of these factors on dog spermatozoa, and it is the purpose of this paper to present similar studies using the spermatozoa of this species.

METHODS

The spermatozoa-bearing fraction of dog semen was collected by manipulation. Only apparently normal specimens of good initial motility were employed, usually pooled with at least one other ejaculate.

The diluents used were glucose and sodium chloride mixtures buffered with: (*a*) citric acid and disodium phosphate, (*b*) mono- and disodium phosphates, (*c*) sodium bicarbonate–carbonate, or (*d*) veronal–HCl. The tables of Kolthoff & Rosenblum (1937) and Vogel (1943) were used as a guide in preparing the buffers, the proportions of the constituents, which were all at a final concentration of 0·02 M, being carefully adjusted to give the desired pH. Details of the composition of the diluents are given in Table 1, the relative tonicity being calculated on the assumption that all electrolytes were completely dissociated. Owing to dissociation into varying numbers of ions the tonicity of the buffer mixtures varied slightly at different pH levels, and this was compensated by adjusting the sodium chloride concentration of the diluent. All diluents were stored in a deep-freeze and the pH checked with a glass electrode meter before and after each experiment. The actual pH of suspensions sometimes differed slightly from the intended pH, particularly towards the end of some experiments, and the extent of this variation is shown in the tables by the standard deviation.

Semen was diluted 1:20 in small tubes for the motility studies which were made at room temperature. Motility was scored by the system of Emmens (1947) at ½, 1½, 2½, 4 and 5½ hr unless otherwise stated. Full motility was rated as 4 and complete immotility as zero, but in presenting results the actual scores have been multiplied by 4, since quarter grades were frequently used.

The motility index referred to in the tables is the sum of the motility scores × 4 for each ejaculate over the experimental period. This has been used as unit observation (see Emmens, 1948) for the analyses of variance which are presented in summary form, the error mean square (residual variance) and variance ratios being shown with asterisks to indicate significant differences in motility.

TABLE 1. Composition of diluents

Diluent	Relative tonicity (0·9 % NaCl = 100)	Glucose (%)	NaCl % at nominal pH 3·9	5·4	7·1	8·6	10·0*
A	50	2·00	0·00	0·00	0·00	0·00	0·00
B	100	2·00	0·46	0·46	0·42	0·46	0·43
C	150	2·00	0·91	0·91	0·87	0·91	0·88
D	200	2·00	1·36	1·36	1·32	1·36	1·33
E	50	1·00	0·17	0·17	0·13	0·17	0·14
F	100	3·75	0·17	0·17	0·13	0·17	0·14
G	150	6·55	0·17	0·17	0·13	0·17	0·14
H	200	9·30	0·17	0·17	0·13	0·17	0·14

* NaOH was added to the veronal–hydrochloric acid mixtures to bring the pH to 10·0.

RESULTS

Preliminary tests

No one buffer would cover the required wide pH range (4–10), and preliminary tests were undertaken to find a combination of equally innocuous buffers. Dog spermatozoa retained high motility in isotonic diluents *B* and *F* containing phosphate buffer, and these were used as standards for the comparison of other buffers. All comparisons were made at a common overlapping pH as close as possible to neutrality. The comparison of citric acid–phosphate *versus* phosphate was made at pH 6·0, that of veronal–hydrochloric acid *versus* phosphate at pH 6·9, and those of veronal–hydrochloric acid *versus* carbonate–bicarbonate and carbonate–bicarbonate *versus* phosphate at pH 8·5. Five ejaculates were used in each test and motility was scored at ½, 1½, 3 and 5 hr from the start of the test.

The results are shown in Table 2 with summary analyses of variance in Table 3. The comparison of citric acid–phosphate *versus* phosphate is not included in Table 2 as motility in each buffer at pH 6·0 was zero. The motility score was significantly lower in carbonate–bicarbonate than in phosphate ($P<0{\cdot}05$). Motility in veronal–hydrochloric acid, however, was quite as good as in phosphate and significantly better than in carbonate–bicarbonate ($P<0{\cdot}01$). The veronal–hydrochloric acid buffer was therefore used in preference to the carbonate–bicarbonate in subsequent tests over the alkaline pH range. It may be noted that both the phosphate and carbonate–bicarbonate buffers tended to be unstable at pH 8·5. Thus, while the mean pH of each buffer at the start of the tests agreed within 0·1 unit, the mean pH of the carbonate–bicarbonate buffer at the conclusion of the 5 hr tests was 8·7 (S.D. = 0·22) and that of the phosphate buffer 8·0 (S.D. = 0·08). This would not, however, be expected to affect seriously the comparison of the buffers, since the pH shift was mainly confined to the last 2 hr of the tests and subsequent

TABLE 2. Motility indices for five dog ejaculates in diluents containing various buffers

		Diluent *B*						Diluent *F*						
pH	Buffer	1	2	3	4	5	Total	1	2	3	4	5	Total	Total
8·5	Carbonate–bicarbonate	23	29	17	25	22	116	30	22	16	54	48	170	286
	Phosphate	41	49	46	62	43	241	42	50	49	39	46	226	467
6·9	Veronal–HCl	43	35	54	48	60	240	39	34	52	47	52	224	464
	Phosphate	47	43	37	51	60	238	52	42	44	50	62	250	488
8·5	Carbonate–bicarbonate	35	23	39	42	59	198	47	29	38	44	63	221	419
	Veronal–HCl	61	45	59	56	64	285	46	49	53	59	63	270	555

TABLE 3. Summary of the analysis of variance for the data in Table 2: the residual variance is shown in italics at the base of each column

		Variance ratios		
Source of variation	Degrees of freedom	Carbonate–bicarbonate *versus* phosphate	Veronal–HCl *versus* phosphate	Carbonate–bicarbonate *versus* veronal–HCl
Between diluents	1	0·5	0·1	0·1
Between buffers	1	11·0*	4·3	30·8**
Between ejaculates	4	0·7	30·9**	11·3*
Interactions:				
Diluent: buffer	1	1·6	5·7	2·4
Buffer: ejaculate	4	0·6	10·9*	0·4
Diluent: ejaculate	4	0·3	0·5	1·6
Residual	4	*149*	*7*	*30*

* $P<0{\cdot}05$. ** $P<0{\cdot}01$.

experiments in isotonic diluents show little difference in motility score over this pH range.

Systematic studies

The motility of dog spermatozoa was studied at five pH levels (nominally 4·0, 5·5, 7·0, 8·5 and 10·0). At each pH level the eight diluents of Table 1 were used, so that at four levels of tonicity the effect of substituting sodium chloride for part of the glucose could be examined. All experiments were repeated five times, each ejaculate (usually pooled specimens) being partitioned between tubes representing all possible combinations of the factors investigated.

TABLE 4. The effect of pH, tonicity, sodium chloride and glucose concentrations on the motility indices of dog spermatozoa

		pH of suspension																		
		5·5±0·1						7·0±0·1						8·3±0·1						
Ejaculates		1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total	Tot
Diluents	Tonicity																			
A	50	2	0	0	2	1	5	10	0	0	0	5	15	1	1	0	24	2	28	4
B	100	0	0	1	1	0	2	78	79	70	63	68	358	75	75	48	73	56	327	68
C	150	5	0	0	0	0	5	40	48	34	64	50	236	52	51	28	64	10	205	44
D	200	1	0	0	0	0	1	16	20	4	46	14	100	19	34	9	63	51	176	27
E	50	0	0	0	0	0	0	38	37	28	52	68	223	3	2	3	0	0	8	23
F	100	4	3	3	7	0	17	77	48	29	75	70	299	69	74	71	62	63	339	65
G	150	0	0	0	0	0	0	75	74	45	64	54	312	76	59	49	23	19	226	53
H	200	0	0	0	1	0	1	61	65	54	2	1	183	44	37	7	25	57	170	35
Totals		12	3	4	11	1	31	395	371	264	366	330	1726	339	333	215	334	258	1479	323

TABLE 5. Summary of the analysis of variance for the data of Table 4 at two pH levels (7·0 and 8·3). The residual variance is given in italics at the base of the variance ratio column

Source of variation	Degrees of freedom	Variance ratio
Between ejaculates	4	2·5
Between pH levels	1	2·7
Between diluents	7	17·1**
Interactions:		
Ejaculate: pH	4	<1
Ejaculate: diluent	28	1·0
Diluent: pH	7	2·9*
Residual	28	*278*

* $P<0{\cdot}05$. **$P<0{\cdot}01$.

The results are set out in Table 4 and illustrated in Fig. 1; the analysis of variance is summarized in Table 5. The motility score was zero with all diluents at pH 4·0 and 10·0 and these pH levels have been omitted from Table 4. Even at pH 5·5 motility was very low, and in order to avoid heterogeneous variances only the motility scores at pH 7·1 and 8·3 have been analysed. Hypotonic diluents were clearly more detrimental than hypertonic diluents at all pH levels. The over-all analysis shows no significant difference between motility at pH 7·1 and 8·3, and dog spermatozoa are much more tolerant to alkaline than to acid conditions, although dog semen normally tends to be

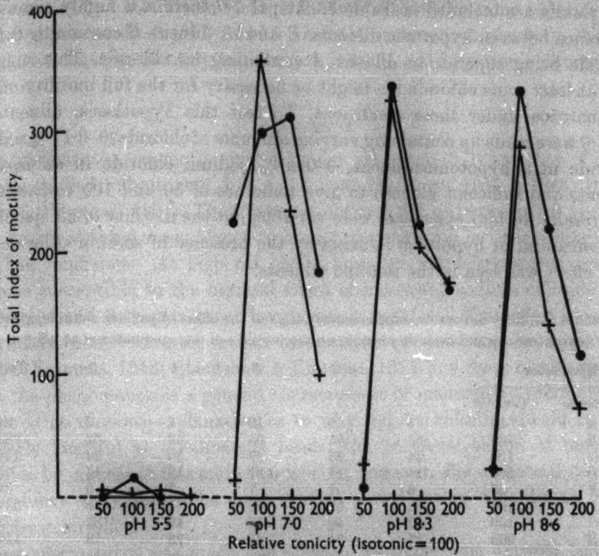

Fig. 1. The interaction of pH, osmotic pressure and electrolyte content on the motility of dog spermatozoa. The mean indices of motility (see text) are taken from Table 5. +—+, constant percentages of glucose, varying amounts of sodium chloride; ●—●, constant percentage of sodium chloride, varying percentage of glucose.

acid. The mean pH of seventeen sperm-bearing fractions was 6·3 (S.D. = 0·23; range = 6·9–5·9). The prostatic fluid with which the spermatozoa are normally mixed, however, has a slightly higher pH; thus the mean for eight samples of prostatic fluid was 6·8 (S.D. = 0·33; range 6·3–7·2).

In view of the highly significant interactions on over-all analysis, separate analyses were made at pH 7·1 and 8·3 according to the following scheme:

	Factorial coefficients for diluents							
Source of variation	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Part replacement of glucose by NaCl, tonicity 50	+1	0	0	0	−1	0	0	0
Part replacement of glucose by NaCl, tonicity 100	0	+1	0	0	0	−1	0	0
Part replacement of glucose by NaCl, tonicity 150	0	0	+1	0	0	0	−1	0
Part replacement of glucose by NaCl, tonicity 200	0	0	0	+1	0	0	0	−1
Curve fitting								
Linear regression	−3	−1	+1	+3	−3	−1	+1	+3
Simple curvature	+1	−1	−1	+1	+1	−1	−1	+1
Double curvature	−1	+3	−3	+1	−1	+3	−3	+1

The results are included in Table 7. At pH 7·0 there is a highly significant difference between hypotonic diluents *E* and *A*, diluent *E* containing 0·13% chloride being superior to diluent *A* containing no chloride. This suggests that at least some chloride ion might be necessary for the full motility of dog spermatozoa under these conditions. To test this hypothesis, diluents at pH 7·0 were made up containing varying amounts of chloride (0–0·17% sodium chloride in a hypotonic diluent, 0–0·68% sodium chloride in an isotonic diluent) and sufficient glucose to give tonicities of 50 and 100 respectively. The results for four ejaculates were variable, but the motility of all ejaculates was enhanced in hypotonic diluents by the presence of sodium chloride; no such effect was seen in the isotonic diluents.

Table 6. Motility indices for supplementary tests of the effect of sodium chloride, glucose concentrations and osmotic pressure on the motility of dog spermatozoa at pH 8·8

Ejaculates ...		1	2	3	4	5	Total
Diluents	Tonicity						
A	50	4	2	10	5	3	24
B	100	55	51	65	66	52	289
C	150	34	25	29	30	24	142
D	200	14	11	21	16	14	76
E	50	6	1	7	7	3	24
F	100	68	64	72	74	59	337
G	150	52	40	45	49	37	223
H	200	27	24	25	23	18	117
Totals		260	218	274	270	210	1232

Table 7. Summary of the analysis of variance at separate pH levels for the data of Tables 4–6. The residual variance is shown in italics at the base of each column

Source of variation	Degrees of freedom	Variance ratios at pH		
		7·1	8·3	8·7
Between diluents				
Glucose *versus* NaCl (tonicity 50)	1	15·1**	0·2	0
Glucose *versus* NaCl (tonicity 100)	1	1·2	0·1	24·5**
Glucose *versus* NaCl (tonicity 150)	1	2·0	0·2	69·8*
Glucose *versus* NaCl (tonicity 200)	1	2·4	0·0	17·9**
Curve fitting	3	43·3**	78·9**	1970·2**
Between ejaculates	4	1·1	1·5	12·1**
Residual	28	*286*	*258*	*9*

* $P<0{\cdot}05$. ** $P<0{\cdot}01$.

In view of the extension of the optimal pH into the alkaline region and at the same time the severe falling off in motility at pH 10·0, further tests were undertaken to check the possibility of a beneficial effect of replacement of sodium chloride by glucose which was seen with the other species at alkaline pH levels (Blackshaw & Emmens, 1951). The veronal–HCl buffer at pH 9·2 was used. The results for five ejaculates are presented in Table 6 and the summary analysis of variance is included in Table 7. The initial pH of the diluents was 8·8 (S.D. = 0·17) after semen addition and the pH after 5½ hr was

8·5 (S.D. = 0·11). Motility in isotonic diluents was of the same order as that observed previously at pH 7·1 and 8·3 and the replacement of sodium chloride by glucose was significantly beneficial in isotonic and hypertonic diluents.

DISCUSSION

Dog spermatozoa are motile over a similar pH range (5–10) to that of other species studied, rabbit, ram, bull and human (Emmens, 1947 & 1948; Blackshaw & Emmens, 1951). They have an optimal pH of between 7·0 and 8·5, and bear a striking resemblance to human spermatozoa in their tolerance to alkaline conditions. At high pH levels, however, dog spermatozoa tend to become susceptible to the harmful effect of increasing sodium chloride concentration and its replacement by glucose is beneficial. This effect has been previously observed with other mammalian spermatozoa in hypertonic media (Emmens, 1948; Blackshaw & Emmens, 1951) and these results suggest that the phenomenon is a general characteristic of mammalian spermatozoa. There is no obvious explanation as to why sodium chloride should be particularly harmful at alkaline pH levels; the beneficial action of replacing chloride by glucose in this and other work is, however, due to the maintenance of viability throughout the experimental period rather than to an initial increase in motility.

Dog spermatozoa are much less affected by hypertonic diluents than by hypotonic ones of similar composition. They resemble ram, bull and human in their response to changes in tonicity (Blackshaw & Emmens, 1951) and do not show the interaction between pH and tonicity characteristic of rabbit spermatozoa (Emmens, 1948).

Dog spermatozoa are apparently unique among the species which have so far been studied in that their motility is depressed by diluents containing 0·02 M carbonate–bicarbonate buffer. No such effect was seen with rabbit spermatozoa previously (Emmens, 1947). It may be noted, however, that VanDemark & Sharma (1957) have used a medium containing 0·2 % sodium bicarbonate saturated with CO_2 to immobilize bull spermatozoa temporarily. These can then be kept at room temperature for several days without loss of fertility.

SUMMARY

1. The motility of dog spermatozoa has been studied *in vitro*, under various modifications of pH, osmotic pressure and chemical composition of diluents.

2. The glucose and sodium chloride contents of the diluents have been varied to give tonicities ranging between those of 0·45 and 1·8 % sodium chloride. These diluents were buffered with citric acid–phosphate, sodium phosphates, sodium carbonate–bicarbonate or veronal–hydrochloric acid mixtures.

3. The sodium carbonate–bicarbonate buffer mixture depressed the motility of dog spermatozoa, but the other buffers were equally innocuous.

4. Dog spermatozoa showed maximum motility in the 7·0–8·5 pH range. The pH of the sperm-bearing fraction of dog semen was 6·3 ± 0·23 and the prostatic fluid 6·8 ± 0·33.

5. At a pH of 8·7–8·9 partial replacement of sodium chloride by glucose was favourable in isotonic and hypertonic diluents.

6. Under all conditions hypotonic diluents were more deleterious than hypertonic ones of similar composition.

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THE EFFECTS OF HEXAMETHONIUM BROMIDE ON TEMPERATURE REGULATION IN MAN

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The actions of autonomic blocking agents on temperature regulation of rats can be accounted for by the effects of increased heat loss subsequent to peripheral vasodilatation (Grayson & Mendel, 1956) and of reduced heat production due to blocking of 'chemical regulation' (Hsieh, Carlson & Gray, 1957). The latter effect is not important since 'chemical regulation' is significant only in cold-acclimatized rats and when muscle activity is blocked by curare (Cottle & Carlson, 1956). In man, increased skin temperature after sympathetic blockade with hexamethionium bromide has been reported (Finnerty & Freis, 1950). Hamilton, Henley & Morrison (1954) have suggested that increased heat loss from dilated vessels of the skin, mainly of the feet, may be responsible for the fall in body temperature following parenteral hexamethonium. The experiments of Hamilton *et al.* were performed on subjects comfortably warm. This paper reports experiments on two subjects, exposed to moderately low room temperature (20° C), in which oxygen consumption and regional skin temperatures were measured before and after intramuscular injection of hexamethonium bromide.

METHODS

The experiments were conducted before breakfast with the subjects lying nude in a room maintained at 20° C. The temperatures of the head, arms, hands, feet, legs, thighs and trunk were recorded by copper-constantan thermocouples taped to the skin and located at the points recommended by Hardy & DuBois (1938) whose formula was used for calculating the average skin temperature. Rectal temperature was recorded with a copper-constantan thermocouple probe inserted about 6 in. (15·2 cm). In calculating the mean body temperature the formula used was: mean body temp. $=0\cdot65\times$(rectal temp.)$+0\cdot35\times$(average skin temp.) (Burton, 1935). Oxygen consumption was determined by measuring the inspiratory volume for 5 min periods and analysing samples of the expired air with a Pauling-Beckman oxygen-analyser (modified from Margaria, Meschia & Marro, 1954). Oxygen tension was read to the nearest 0·5 mm Hg.

Recordings were begun 30 min after the subject had removed his clothes and lain down on the bed. Four experimental procedures were followed: (*a*) control experiments in which the subjects lay quietly for 140 min; (*b*) after a control period of 50 min, 100 mg of hexamethonium bromide

(Vegolysen, May and Baker) was injected intramuscularly and the response followed for 90 min; (*c*) the effects on the response to hexamethonium produced by blocking the circulation to both feet for 20 min were tested by inflating to 260 mm Hg sphygmomanometer cuffs wrapped around the ankles; (*d*) the effects of reducing the heat loss from the feet on the response to hexamethonium were tested by keeping the feet wrapped in cotton-wool throughout the experimental period.

Each experiment was performed twice on the same subject. Tolerance to hexamethonium is assumed not to have occurred since the experiments were performed at intervals of at least 4 days.

RESULTS

Control experiments

With the subjects lying quietly at 20° C for 140 min a gradual fall of skin temperature occurred, most marked in the hands (6·3° C) and feet (7·4° C) and least in the head, trunk and thighs. The arms and legs showed an intermediate rate of fall, being about 4·6° and 2° C respectively during the period of recording (Fig. 1).

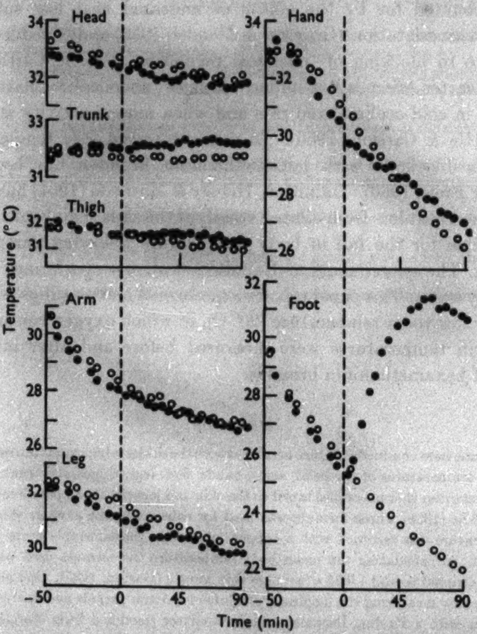

Fig. 1. Skin temperature of various regions of the body of nude subjects lying quietly at 20° C room temperature. ○, results from control experiments; ●, experiments in which 100 mg hexamethonium bromide was injected intramuscularly at zero time. Each point is the average of four experiments performed on two subjects.

The steady fall of foot temperature suggests that vasoconstriction was complete by the time recordings were begun. This assumption was tested in separate experiments in which cuffs wrapped around the ankles were inflated to 260 mm Hg for 15 min periods. Foot temperature continued to fall at the same rate during occlusion. After releasing the pressure in the cuffs a rise in temperature took place, coincidental with 'reactive hyperaemia', and reaching a maximum in 5 min; it then continued to fall at the previous rate (Fig. 2).

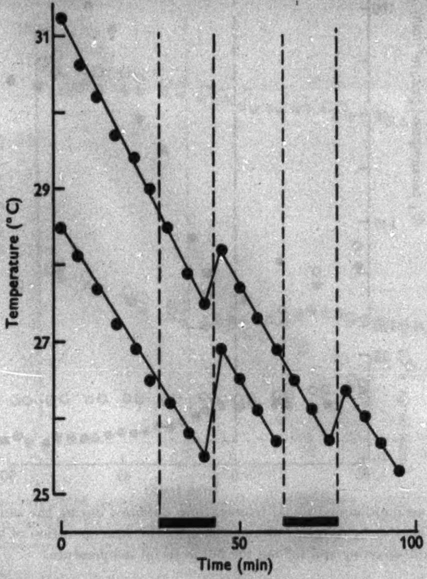

Fig. 2. The temperature of the feet of nude subjects lying quietly at 20° C room temperature. During the periods indicated by the horizontal bars, occlusion cuffs placed around the ankles were inflated to 260 mm Hg for 15 min.

Very little change in rectal temperature or in oxygen consumption took place during the test period (Fig. 3). During the last half hour of exposure the subjects experienced occasional muscle tremors over the trunk which were described as shivering. Assuming a caloric value of 4·82/l. O_2, the mean metabolic rate for the subjects was 36·2 kcal/m^2/hr.

The calculated average skin temperature was about 31·6° C at the beginning of the experiment and 29·7° C 140 min later (Fig. 4). When the temperatures of the hands and feet were omitted from the calculations the reduction in skin temperature was about 1° C. Thus the hands and feet, possessing only about

12% of the body surface area, were responsible for about 50% of the fall in the average skin temperature. The mean body temperature was 35·4° C at the beginning of the experiments and 34·6° C at the end.

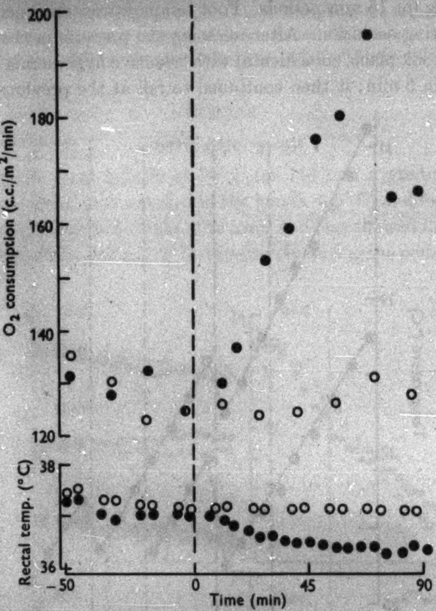

Fig. 3. Oxygen consumption and rectal temperatures obtained during the same experiments as in Fig. 1. The rise in oxygen consumption after the administration of hexamethonium is associated with shivering and follows the fall in rectal temperature.

Effects of hexamethonium

The temperature changes of the head, arms, legs and hands were not significantly altered after the injection of hexamethonium. Small increases in the temperature of the trunk and thighs were recorded, but as these were associated with vigorous shivering of the muscles in these areas it is possible that they were not due to direct action of the drug. The temperatures of the various regions are shown in Fig. 1, for comparison with the control readings.

The most striking change was the rise in the temperature of the feet. The response usually appeared within 5 min of the injection and reached a maximum in about 60 min. The maximum to which the temperature of the feet rose was fairly constant, being about 32·3° C. As the initial temperatures varied between 24·2° and 25·9° C, the magnitude of the rise was determined by the initial temperature of the feet. The average rise in foot temperature was about 5·7° C.

Soon after the temperature of the feet started to rise, the rectal temperature began to fall. The fall in rectal temperature was initially rapid, being about 0·5° C during the first 30 min after the injection. Thereafter, the rectal temperature remained at approximately the same level for the rest of the experimental period (Fig. 3).

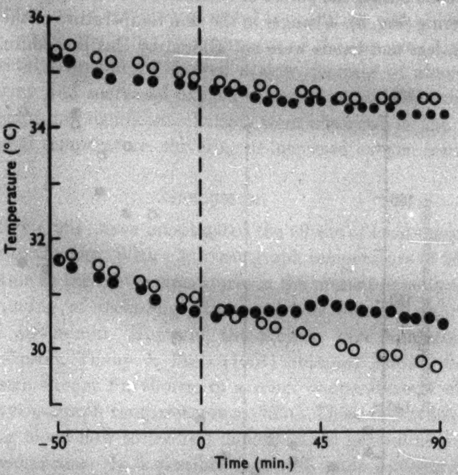

Fig. 4. Upper line, calculated mean body temperatures; lower line, average skin temperatures; obtained from the data for skin temperatures shown in Fig. 1, and rectal temperatures in Fig. 2. The mean body temperature is not altered by the injection of hexamethonium (upper solid circles).

The subjects began to shiver about 7 min after the injection of hexamethonium. Shivering started as occasional tremors of the intercostal muscles and became generalized in about 30 min. The occurrence of shivering was not associated with any feeling of coldness; on the contrary, the subjects felt warmer than before the injection of the drug. Oxygen consumption increased when shivering occurred, the increase being parallel to the vigour of the shivering (Fig. 3).

The calculated average skin temperature showed a slight rise above pre-injection levels, in contrast to the gradual fall observed in the control experiments. No significant difference was observed between the mean body temperature in the control experiments and those in which hexamethonium was injected (Fig. 4).

Effects of occlusion of blood flow to the feet

When the temperature of the feet began to show a reduced rate of fall, cuffs applied around the ankles were inflated to about 260 mm Hg for 20 min. During the period of occlusion there was no drop in rectal temperature and shivering did not occur. Release of the pressure in the cuffs was followed by a rapid fall in rectal temperature and shivering. Oxygen consumption, which was not elevated during the period of occlusion, rose sharply with the appearance of shivering (Fig. 5). Changes in the skin temperature of the head, trunk, thighs, arms, legs and hands were not altered by this procedure.

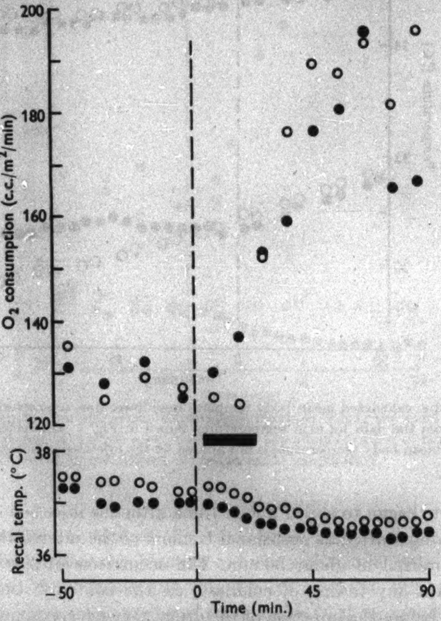

Fig. 5. Oxygen consumption and rectal temperature of subjects lying nude at 20° C room temperature. At zero time 100 mg of hexamethonium bromide was injected intramuscularly. In the experiments indicated by the open circles, sphygmomanometer cuffs applied around the ankles were inflated to 260 mm Hg for a period of 20 min, indicated by the horizontal bar. The solid circles are replots from Fig. 2, and are shown for comparison.

Effects of reduced heat loss from the feet

Wrapping the feet in cotton-wool reduced the rate of fall of temperature in the feet. The changes in the skin temperatures of the other regions were not altered by this procedure. The temperature of the feet at the time of injection of hexamethonium varied greatly, ranging from 29·9 to 34·5° C, but the maximum temperatures reached after injection varied by only ±0·3° C from the mean of 35·2° C. The rise in foot temperature ranged from 0·8 to 5·1° C.

The only alteration in response of rectal temperature was observed in one experiment in which the initial temperature of the feet was high. In this experiment rectal temperature showed a slow decrease of about 0·3° C in 90 min; shivering and increased oxygen consumption did not occur. In the other experiments no significant changes were observed in the pattern of response in rectal temperature, shivering or increased oxygen consumption.

DISCUSSION

Allwood & Burry (1954) have investigated the effects of local temperature on the blood flow to the feet. With plethysmograph temperature of 26° C, which is about the same as the foot temperatures in the present experiments 50 min after the beginning of recordings, these workers obtained values of 0·53–1·3 ml./100 ml. tissue/min. However, the subjects were comfortably warm. Schnapper, Johnson, Tuohy & Freis (1951) obtained similar results from subjects exposed, except for shorts, to a room temperature of about 20° C but with plethysmograph temperatures at 32° C. These observations suggest that the resting blood flow to the feet depends upon general as well as upon local reflex mechanisms. It is possible that with nude subjects exposed to a room temperature of 20° C the blood flow to the feet may be less than those shown in published figures. In the present experiments, the fact that inflation of occlusion cuffs placed around the ankles did not increase the rate of fall of foot temperatures strongly suggests that vasoconstriction is complete early on exposure to cold. Pressure on the nerves during the period of inflation may have resulted in a reduction in the vasomotor tone of the vessels in the feet. This could explain the appearance of 'reactive hyperaemia' after the release of the pressure from the cuffs. It may therefore be assumed that loss of body heat through the feet was very small during the control periods.

The appearance of shivering after about 2 hr exposure to moderate cold is a common observation. In the present control experiments, shivering appeared without any change in rectal temperature. This suggests that the stimulus for shivering was the alteration in skin temperature. The occurrence of shivering after such long delay might depend on the ability of the cold receptors to indicate absolute temperature as well as the rate of temperature change (Hensel, 1952), and also the summation of impulses in the temperature regulation centre, as suggested by Carlson (1954). Recent work supports this hypothesis (Spurr, Hutt & Horvath, 1957).

The rapid fall in rectal temperature and the occurrence of shivering after the injection of hexamethonium cannot be explained by assuming increased heat loss from the body subsequent to a rise in skin temperature. The average skin temperature was not altered at the time these changes appeared. Furthermore, reducing the rate of heat loss from the feet did not alter significantly the course of events. The results can, however, be explained by assuming an alteration in the distribution of the heat in the body. The fact that the calculated mean body temperature was not changed by the injection of hexamethonium supports this suggestion. The marked rise in the skin temperature of the feet after hexamethonium indicates that body heat may be shifted to this region. It is known that blood flow to the feet is greatly increased after administration of hexamethonium (Schnaper *et al.* 1951). Blood entering a cold extremity would lose heat to the tissues and return at a lower temperature (Bazett, Love, Newton, Eisenberg, Day & Foster, 1948). This cooled venous blood could produce a drop in rectal temperature and initiate shivering in the manner suggested by Uprus, Gaylor & Carmichael (1935). The possibility that shivering was due to the direct action of hexamethonium is ruled out by the finding that shivering did not occur in the absence of a fall in rectal temperature, or when the rate of fall in rectal temperature was slow.

Lack of information as to the weight of the feet, the specific heat of the tissues of the feet and the exact temperature changes, renders it impossible to calculate accurately the amount of heat necessary to produce the observed average rise of 5·7° C in the skin temperature of the feet. However, by assuming 0·83 kcal/kg/° C for the specific heat, 1·8 kg for the weight of the feet and 5·7° C for the temperature difference, a value of 8·5 kcal is obtained. Removal of this amount of heat from the blood circulating through the feet during the 60 min period of temperature rise can account for the observed fall in rectal temperature. The subsequent steady state with the rectal temperature at a lower level can be explained by assuming that the increased heat production from shivering balances the extra heat lost from: (*a*) increased convection that occurs with shivering and (*b*) the increased surface area for heat dissipation that occurs when the blood re-enters the feet.

Hamilton *et al.* (1954) did not observe shivering or increased oxygen consumption after injection of hexamethonium. In experiments on nude subjects at room temperatures of 26° C Hsieh (unpublished) obtained similar results. The average skin temperatures of the subjects in these experiments were higher and the rectal temperatures fell at a slower rate. The higher average skin temperature could result in an inhibition of shivering (Jung, Doupe & Carmichael, 1937). In these circumstances it is possible that increased heat loss due to reduction in insulation of the body may play an important role in the fall of deep body temperature that occurs after administration of hexamethonium. However, the rise in foot temperature is still considerable (as much as 4° C) and heat lost from the blood to the tissues of this area cannot be ignored.

Hsieh *et al.* (1957) observed a reduction in the oxygen consumption of curarized rats after intravenous injections of hexamethonium and suggested that the drug might act by blocking sympathetic control of chemical regulation of heat production. Oxygen consumption was not reduced by the administration of hexamethonium in the present experiments. Others (Reynolds, Paton, Freeman, Howard & Sherlock, 1953; Hamilton *et al.* 1954) have made similar observations. It may well be that muscular activity in the form of increased muscular tone may still be present even with subjects lying quietly. This would mask any reduction in 'chemical regulation'. Another possibility is that the dose of hexamethonium may have been too small.

SUMMARY

1. The effects of intramuscular injection of hexamethonium bromide on temperature regulation in man have been investigated by following the changes in skin temperature, rectal temperature and oxygen consumption in two male subjects when lying nude in a room maintained at 20° C.

2. Skin temperatures of the head, arms, legs and hands are not altered by the injection of hexamethonium. There are small increases in the temperatures of the trunk and thighs, but these are attributable to the associated shivering in these areas.

3. When sphygmomanometer cuffs placed around the ankles are inflated to about 260 mm Hg soon after the injection of hexamethonium, and the pressure is maintained for 20 min, the above changes do not appear until the pressure is released.

4. The effects of hexamethonium on temperature regulation in the present experiments have been explained on the basis of increased blood flow through the feet and the thermal state of the feet at the time of the administration of this drug.

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SOME EFFECTS PRODUCED BY ADRENALINE UPON NEUROMUSCULAR PROPAGATION IN RATS

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Some fifty years have passed since the first reports of a potentiating action of suprarenal extracts on the contractions of fatigued muscle (Dessy & Grandis, 1904; Panella, 1907). This subject has inspired a voluminous literature, which has been conspicuous for the variety of phenomena described and the diversity of explanatory hypotheses. A historical survey is out of the question, but reference may be made to four authors who give useful reviews covering the greater part of the field: Gruber (1919); Wastl (1928); Burn (1945) and Goffart (1952).

A study of this literature gives a somewhat confusing impression, mainly because of the paucity of well established facts. It is, for instance, still not agreed whether adrenaline has an action of any importance on neuromuscular propagation (cf. Brown, Bülbring & Burns, 1948); or whether adrenaline really alters the membrane potential of muscle fibres (cf. Brown, Goffart & Vianna Dias, 1950; Hutter & Loewenstein, 1955). With this picture in mind it seemed to us that a detailed investigation of the effects of adrenaline at various critical points in neuromuscular propagation should provide a concrete answer to some fairly simple questions.

We have suggested in a previous publication (Krnjević & Miledi, 1958*b*) that two main types of failure of neuromuscular propagation are associated with fatigue. Post-synaptic failure, which commonly tends to occur first, is characterized by the failure of initiation of conducted impulses in the muscle fibres. It is apparently caused by two principle factors: a rise in the threshold of the fibre; and a reduced sensitivity of the end-plate to ACh. Presynaptic failure of conduction, on the other hand, prevents impulses from reaching the nerve ending. We have now studied the effects of adrenaline on these kinds of failure in rat muscle both *in vitro* and *in situ*. We have also looked for alterations in the resting potential of muscle fibres.

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* Fellow of the Rockefeller Foundation.

Like so many earlier authors, we have found the actions of adrenaline to some extent unpredictable. Nevertheless, certain trends can be clearly discerned. There is no doubt that there is a powerful defatiguing effect on neuromuscular propagation. A preliminary account has been given of some of the observations described here (Krnjević & Miledi, 1957).

METHODS

All muscles were from albino rats of Wistar origin, weighing 100–250 g. The recording and stimulating techniques were similar to those previously described (Krnjević & Miledi, 1958*b*).

Experiments in vitro

In twenty-five experiments a phrenic-diaphragm preparation was mounted in a Perspex chamber with a capacity of about 20 ml. The bathing Ringer-Locke solution had the composition given by Liley (1956*a*); it was stirred vigorously with a mixture of 5% CO_2 in O_2, and was kept either at room temperature (about 22° C) or at 37° C. The proximal portion of the phrenic nerve rested on platinum stimulating electrodes in a separate compartment filled with liquid paraffin. The electrical activity of single muscle fibres was recorded with conventional intracellular glass micro-electrodes filled with 3 M-KCl. For direct electrical stimulation we used a non-polarizable electrode (Ag–AgCl–Agar–Ringer) with a diameter of 0·7 mm. The muscles were usually fully paralysed with D-tubocurarine chloride (Burroughs Wellcome and Co.).

Artificial end-plate potentials were obtained by applying ACh (acetylcholine chloride, Roche Products) electrophoretically with micropipettes. The square pulses of current had a duration of 1–10 msec, and a magnitude of about 10^{-7} A. The concentration of ACh in the glass micropipettes was 2–4 M. Spontaneous diffusion was controlled by an adjustable inward current.

Injection of adrenaline. In the earlier experiments a continuous flow of Ringer-Locke solution was maintained through the muscle chamber. Adrenaline was injected into the system at a convenient point upstream from the chamber. In most experiments the bathing solution was only changed whenever it was necessary to wash out expended adrenaline, etc. The drug was then injected directly into the chamber, either from a burette, or from a tuberculin syringe via a fine polythene catheter. This method of direct injection is preferable because there is little lag if stirring is adequate, and the final concentration is easily calculated.

Experiments in situ

Five rats were kept under pentobarbitone anaesthesia (Nembutal, Abbott Laboratories) with doses of 5 mg/100 g body weight injected intraperitoneally at intervals of about 1 hr. The left gracilis anticus was exposed and its nerve cut and mounted on platinum stimulating electrodes in a pool of liquid paraffin.

Injection of adrenaline. A fine polythene cannula (PE 10, Clay-Adams, New York) was inserted into the right iliac artery so that its tip lay close to the bifurcation of the aorta. The tubing was filled with Ringer-Locke solution containing heparin. It was connected to a stopcock which permitted the injection of either an adrenaline or a flushing solution. The volume of adrenaline solution injected was of the order of 0·1 ml., in a concentration of 50 μg/ml. We soon found that there is a strict limit to the amount of adrenaline which can be injected into the blood stream without causing pulmonary oedema: according to Maire & Patton (1956), the threshold lethal dose in unanaesthetized rats is 12·5 μg/100 g body weight. We tested the effectiveness of this method of injection with small volumes of a solution of Evans Blue (1/1000). The dye was easily discernible with a microscope when it reached the vessels in the muscle. In preparations with a good circulation the dye appeared in the muscle with a lag of only 1–3 sec.

Adrenaline solutions

As a rule, we used 1/1000 adrenaline hydrochloride (D.H.A. Laboratories, Sydney). In control experiments, the solutions were prepared from the salt adrenaline bitartrate (British Drug Houses). In a few experiments, we also tried the effect of L-noradrenaline bitartrate (Lights and Co. London).

RESULTS

Presynaptic failure of neuromuscular propagation

Prolonged repetitive maximal stimulation of a motor nerve is usually associated, sooner or later, with a failure of conduction somewhere near the nerve terminals, probably at a point of branching. If one records the electrical activity of a single muscle fibre in the region of the end-plate, intermittent failures are observed, which may become more frequent with time, especially at high rates of stimulation (Figs. 1, 3). In some cases, complete and apparently irreversible block may result; more often, the changes are easily reversed by lowering the frequency, or stopping the stimulation altogether.

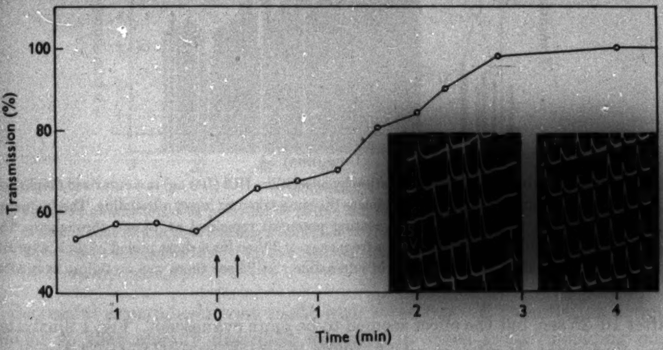

Fig. 1. Complete reversal by adrenaline HCl (60 μg) of intermittent presynaptic failure as recorded intracellularly from the end-plate of a muscle fibre stimulated indirectly at the rate of 10/sec. Abscissa: time after injection of adrenaline, the beginning and end of which are indicated by the arrows; ordinates indicate the corresponding percentage rate of transmission, 10/sec being taken as 100%. Each point is the mean rate of transmission over a period of 24 sec; at 10 min the rate of transmission was still 100%. Diaphragm at 25° C. Inset: examples of the potentials recorded (*A*) before and (*B*) 5 min after adrenaline.

Experiments in vitro. We have found that adrenaline has a potent action tending to restore conduction in fibres which are failing intermittently or, sometimes, even completely. With experiments *in vitro* this was plainly evident in eighteen out of thirty trials. In six cases there was a rather small effect, the significance of which could not be established. In five trials there was clearly no change, and in one case there seemed to be an actual depression of propagation.

The amount of change produced by adrenaline was highly variable. There might be only a very small increase in the number of transmitted impulses, or full conduction might be restored. When the latter happened, it was often possible to raise appreciably the frequency of stimulation without causing further failures (Fig. 2; cf. also Fig. 2, Krnjević & Miledi, 1957). At room temperature there was relatively little change within the first minute of injection and the effect did not reach its peak until 2–3 min had elapsed. Moreover, it often persisted for some 10–15 min even after replacing the solution in the bath. At 37° C recovery of transmission was first observed after 10–25 sec, but the effect seemed to be more evanescent. Fig. 1 illustrates an experiment *in vitro* in which 100% conduction was restored by 60 μg adrenaline HCl. The fibre had been stimulated at a frequency of 10/sec, and was failing intermittently. The electrical activity recorded with an intracellular electrode consisted initially of intermittent spikes. Five minutes after the injection of adrenaline the presynaptic failures no longer occurred. It should be noted, however, that post-synaptic failure was now evident, many impulses failing to evoke anything more than a non-propagated e.p.p.

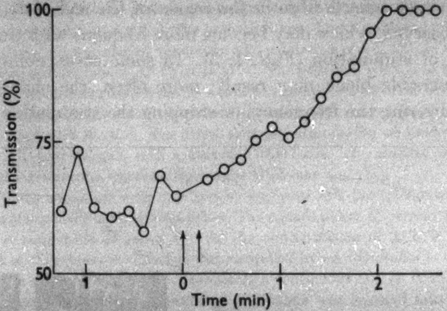

Fig. 2. Restoration of presynaptic conduction by adrenaline HCl (100 μg) in a curarized diaphragm at room temperature. The arrows indicate the time taken to inject adrenaline. The frequency of stimulation was 25/sec and the resting potential remained at 74 mV throughout. The system could follow without failure a frequency of 30/sec for a short period of time, approximately 3 min after the application of adrenaline. At 25/sec there was no failure even after 10 min.

Presynaptic failure of conduction occurs even when the muscular contractions are prevented by curare. Fig. 2 shows that under these conditions adrenaline is no less effective in improving conduction. The contractions can also be much reduced by dissecting and stimulating only a single fibre of the phrenic nerve (Krnjević & Miledi, 1958*a*). Repetitive stimulation readily elicits presynaptic block in such a preparation, and here again adrenaline produces a marked improvement in conduction.

Similar results were obtained with solutions of adrenaline bitartrate. Nor-adrenaline was only administered in one experiment. In two trials better conduction resulted; in two others (one fibre) there was apparently depression.

Experiments in situ. Clear-cut results are less easily obtained with a gracilis *in situ*. Presynaptic failures cannot usually be recognized with certainty unless one records the activity of end-plates with intracellular electrodes. Adrenaline always has a powerful vasoconstrictor action, which causes the muscle to shrink suddenly, in most cases displacing the micro-electrode out of the muscle fibre, and often into another one. This may give a false impression that the nerve fibre is firing at a higher or lower rate. We have only considered experiments in which it was quite clear that the electrode had remained in the same fibre throughout.

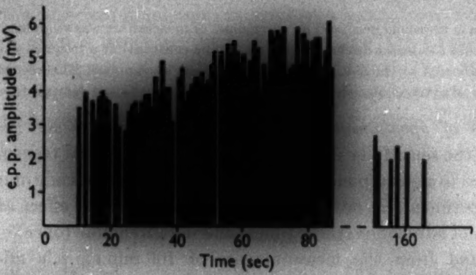

Fig. 3. Effect of adrenaline on presynaptic failure and on e.p.p. amplitude in a curarized gracilis *in situ*. About 10 min after starting stimulation at 1/sec complete presynaptic failure occurred. At zero time adrenaline HCl (2 μg) was injected. Responses first appeared 10 sec later. Each bar represents one e.p.p.

Successful experiments were made with three rats. A potentiating action similar to that already described was seen in seven trials (including one doubtful response). There was no effect in one case, and an apparent depression in another. Fig. 3 shows the return of propagation to a fibre of a curarized gracilis, which had ceased to give e.p.p.'s before adrenaline was injected; e.p.p.'s reappeared 10 sec after the intra-arterial injections, and did not vanish again for nearly 3 min. In all experiments *in situ* the effect had a latency of only 10–25 sec.

Post-synaptic failure of neuromuscular propagation

Sensitivity of the post-synaptic membrane to ACh

Amplitude of ACh-potentials. We have been unable to find any effect of adrenaline upon potentials evoked directly at end-plates in the diaphragm with microjets of ACh at rates of 0·2–5/sec. Various kinds of potentials were produced: some with short (1 msec) pulses; others with longer (10 msec) pulses of ACh. In several cases the adrenaline was added to the bath only after repetitive stimulation had caused the ACh potential to diminish greatly (Fig. 8). Adrenaline and noradrenaline consistently failed to have any significant effect. In two cases the potentials increased somewhat but the control potentials had not been sufficiently regular to make these observations significant, especially in view of the total lack of corroboration in other experiments.

Fig. 4. Increase in threshold produced by adrenaline. A muscle fibre was stimulated directly with just-threshold condenser discharges (0·1 msec time constant) at a frequency of 1/sec; at the moment indicated by the dot, adrenaline HCl (40 μg) was applied. This record was obtained with a pair of extracellular electrodes from a diaphragm at room temperature.

Amplitude of spontaneous miniature end-plate potentials (*min.e.p.p.'s*). A change in the sensitivity of the end-plate to the chemical transmitter ought to be reflected in a corresponding change in the mean amplitude of min.e.p.p.'s. In a few experiments *in vitro* we have looked for such an effect after the administration of adrenaline (three times) and noradrenaline (once). Each mean was calculated from the measurements of 100 min.e.p.p.'s after suitable magnification. In a typical experiment, at room temperature, the *mean* amplitudes were as follows: control, over a period of about 1 min, 1·11 mV; over a period of about 1 min, beginning 1·5 min after the addition of 100 μg noradrenaline to the bath, 1·10 mV.

Changes in the electrical threshold of muscle fibres (*in vitro*)

The first procedure consisted in stimulating a small group of muscle fibres directly with non-polarizable electrodes, at a rate of about once per second. The diaphragm was curarized in some cases; in all cases the stimulating electrodes were placed in a region distant from end-plates. The activity of single fibres was recorded with extracellular electrodes to avoid damage. The intensity of stimulation was adjusted to a level near the threshold, so that some stimuli failed to excite the fibre. Under these conditions, extremely small changes in threshold are readily observed.

A few trials failed to produce any effect. In most cases, however, adrenaline (in doses varying from 10 to 60 μg) had an unmistakable depressing action on the excitability of the fibre (Fig. 4). The change usually began within 10–20 sec, but in some cases there was a delay of some minutes. The period of higher threshold always lasted about 3 min; it was not followed by an obvious overswing.

In the second type of experiment muscle fibres were stimulated directly as before but the rate of excitation was such as to produce a gradual increase in threshold (Krnjević & Miledi, 1958*b*). Adrenaline was then added. This was repeated at several frequencies, and control runs were done at each frequency without adding adrenaline. We found no appreciable difference between the test and the control curves. It must be realized, of course, that this procedure (unlike the first) would not reveal clearly small alterations in excitability (e.g. $<5\%$). We also observed the time course of the recovery of excitability in all cases after ending stimulation. It was unchanged after adrenaline.

Other effects of adrenaline

Effect on e.p.p.'s in muscle fibres paralysed with curare, excess magnesium or fatigue. In the curarized diaphragm we have seen a significant increase in the amplitude of e.p.p.'s (recorded intracellularly) after the administration of adrenaline in a majority of trials (eight out of fifteen). By 'significant' is meant either a pronounced change, which is immediately obvious (e.g. Figs. 5, 6) or one which can be demonstrated statistically in terms of the 0·05 probability limit, using Student's *t*-test. In four trials the results also suggested enhancement, but this was not statistically significant. These cases, and the three in which there was apparently no change at all, were associated with unstable resting potentials; these usually lead to substantial variations in e.p.p.'s, which would tend to mask any other changes of small magnitude. Commonly the increase ranged from 20 to 60%; the greatest increase was

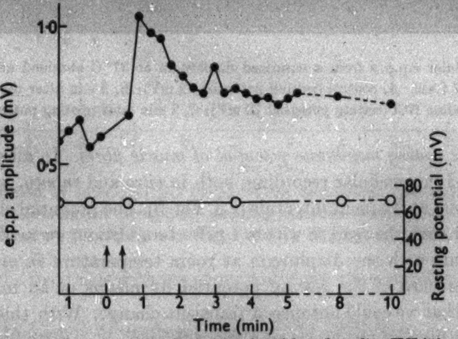

Fig. 5. Enhancement of e.p.p. amplitude (●) produced by adrenaline HCl (50 μg) injected at the time marked by the arrows in a curarized diaphragm at 24° C. Each point corresponds to the mean of 10 responses; the frequency of stimulation was 5/sec. ○, membrane resting potential.

100%. The effect reached its maximum in about 1 min, and to some degree it usually persisted for at least 5 min. We have also found a similar temporary increase of e.p.p.'s in muscles curarized *in situ* (e.g. Fig. 3).

Adrenaline also increases the amplitude of e.p.p.'s in a diaphragm paralysed by a relatively high concentration of magnesium (15 mM). The effect tends to be obscured by the large variations in amplitude which characterize such e.p.p.'s: nevertheless, a significant change was found in three out of five trials. In the other two trials the change was either too small to be significant at the 0·05 level or negligible.

E.p.p.'s in a muscle fibre fatigued by repetitive stimulation are apparently less sensitive to adrenaline. We have only seen an enhancement of amplitude in one case out of four in which we actually looked for such a change (see Fig. 2, in Krnjević & Miledi, 1957).

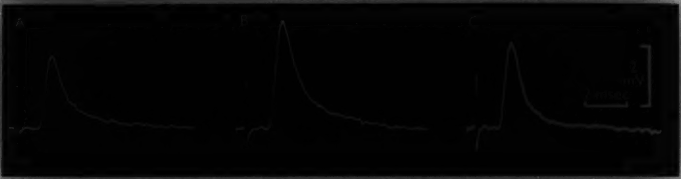

Fig. 6. Intracellular e.p.p.'s from a curarized diaphragm at 37° C obtained at a stimulation frequency of 1/sec. A, control (resting potential 72 mV); B, 2 min after the application of 50 μg adrenaline HCl (resting potential 72 mV); C, 7 min later (resting potential 70 mV).

Effect on the resting membrane potential of muscle fibres. In all experiments which required intracellular recording, both *in vitro* and *in situ*, we noted the resting potential at frequent intervals (e.g. Fig. 5). The potential shown by the millivoltmeter could be read to within 1 mV. In addition, we made a series of five experiments with one diaphragm at room temperature *in vitro*, keeping a continuous record of the resting potential by means of an ink-writer, to make certain that we could not miss a transient change. With this method of recording, the limit of resolution was about 0·5 mV.

We have failed to observe any consistent change of resting potential after the addition of adrenaline. Resting potentials seldom remain steady for a very long time. Usually some drift is evident, more or less random in direction when fibres are not injured. There was no reason to believe that adrenaline added anything to the spontaneous changes already taking place. In a few cases the resting potential varied by less than 1 mV during a period of 6–8 min, near the beginning of which adrenaline had been applied.

Effect on the frequency of spontaneous miniature end-plate potentials. Adrenaline regularly accelerated the discharge of min.e.p.p.'s *in vitro*, both at room temperature and at 37° C (eight trials in five diaphragms). The maximum change resulted in a frequency not much greater than twice the initial average value. Fig. 7 shows the effect on the spontaneous discharges recorded *simultaneously* at two different junctions. The time courses of the changes in the two fibres were remarkably similar, even though the spontaneous discharges had differed substantially, and varied independently of each other, during the preliminary control period.

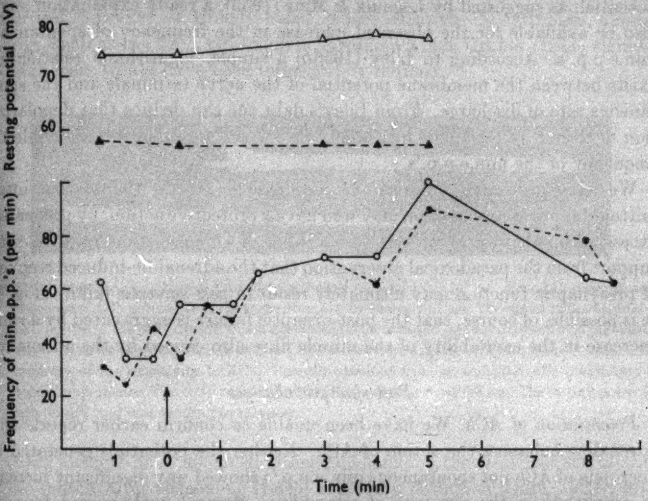

Fig. 7. Acceleration by adrenaline HCl (50 μg) of the spontaneous discharges of min.e.p.p.'s observed by simultaneous intracellular recording from two separate end-plates, ● and ○. ▲ and △ illustrate the respective resting potentials. Diaphragm at 20° C.

In one of the two trials with noradrenaline there was a significant increase in frequency.

A greater frequency of discharge followed the administration of adrenaline *in situ* almost as regularly as *in vitro*. There were only two results of doubtful significance out of nine trials in four rats.

DISCUSSION

Reversal of presynaptic failure

The most striking action of adrenaline tending to improve propagation during fatigue is the presynaptic recovery of conduction in motor nerve fibres (cf. Corkill & Tiegs, 1933). We can do no more than speculate on the mechanism involved. It has been claimed that the electrical threshold of nerve fibres may be diminished under the action of adrenaline (Bülbring & Whitteridge, 1941; Legouix & Minz, 1953). This is supported to some extent by the observation that adrenaline may lower the mechanical threshold of tactile receptors and Pacinian corpuscles (Loewenstein, 1956; Loewenstein & Altamirano-Orrego, 1956), but was not confirmed by Naess & Sirnes (1953), who studied rabbit nerves.

Should the change in threshold really be caused by a reduction in membrane potential, as suggested by Legouix & Minz (1953), a ready explanation might also be available for the observed increase in the frequency of spontaneous min.e.p.p.'s. According to Liley (1956*b*) a simple, logarithmic relationship exists between the membrane potential of the nerve terminals and the spontaneous rate of discharge. From Liley's data one can deduce that depolarization of the nerve terminals by about 5 mV would be sufficient to double the frequency of the min.e.p.p.'s.

We have previously pointed out (Krnjević & Miledi, 1958*b*) that intermittent failure of propagation may well have a protective action, by preventing excessive bombardment of the muscle fibre. This suggestion receives some support from the paradoxical observation that the adrenaline-induced recovery of presynaptic function may ultimately result in *post-synaptic* failure (Fig. 1). It is possible, of course, that the post-synaptic failure is aggravated by a small decrease in the excitability of the muscle fibre also caused by the adrenaline.

Post-synaptic changes

Potentiation of ACh. We have been unable to confirm earlier reports that adrenaline enhances the action of ACh. Neither the potentials generated by microjets of ACh nor spontaneous min.e.p.p.'s showed any significant increase in amplitude in the presence of adrenaline. Several authors had found a depression of the action of ACh on muscle *in situ* (Frank, Nothmann & Guttmann, 1923; Bender, 1938). Luco (1939), however, demonstrated the importance of vascular changes *in situ* (cf. Wastl, 1928), which are apparently responsible for the observed depression. When this is suppressed by ergotoxine, the potentiation is revealed. This is in agreement with the observations of Dale & Gaddum (1930), Burn (1945), Torda & Wolff (1946), Ellis & Beckett (1955) and Hutter & Loewenstein (1955), which were based mostly on experiments with muscles *in vitro*.

It is essential to realize that, with the exception of Hutter & Loewenstein, all these authors measured the contractions or contractures produced by ACh. Their results, therefore, do not permit one to distinguish between a potentiation of ACh and a direct enhancement of the contractile mechanism of the muscle (cf. Goffart & Ritchie, 1952).

Hutter & Loewenstein (1955), on the other hand, demonstrated what seems to be a reversal of the desensitization which follows repeated applications of

ACh. This would be significant during fatigue, one feature of which is reduced effectiveness of ACh (Krnjević & Miledi, 1958*b*). However, in spite of several attempts to produce such an effect, even with relatively large pulses of ACh and high frequencies of release, we have not been able to reverse with adrenaline the progressive diminution of ACh potentials (Fig. 8).

Muscle fibre threshold. The only effect that we could find was a small but distinct increase in threshold during stimulation at a low frequency. There was no evident change produced by adrenaline while stimulating repetitively; the latter test, however, as already noted, is less sensitive. Nevertheless, a large change, such as the 62% reduction in threshold which Gruber (1914) claimed to have seen, would have been immediately noticed.

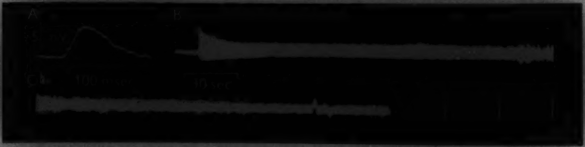

Fig. 8. A, control ACh potential evoked by a 2 msec pulse of $1 \cdot 3 \times 10^{-6}$ A. B and C, a continuous sequence of ACh potentials in the same fibre, at a frequency of 2/sec. The signal at the beginning of C indicates the time during which adrenaline bitartrate (60 μg) was injected into the muscle bath. After ending stimulation at 2/sec, test pulses were applied every 30 sec to illustrate the recovery of the sensitivity to ACh. Records obtained from an end-plate of a diaphragm at room temperature. The time constant of the R.C. amplifier was 0·3 sec. The resting potential was 75 mV and was tending to increase.

Other authors have observed either no change in the electrical excitability of the muscle after adrenaline (Luco, 1939) or a reduction (Obré, 1923); even Gruber (1922) later found depression in denervated muscle.

Other effects of adrenaline

Decurarizing action. The enhancement by adrenaline of neuromuscular transmission blocked by curare is now too well substantiated to be much in doubt (Gruber, 1914; Rosenblueth, Lindsley & Morison, 1936; Hutter & Loewenstein, 1955), in spite of the negative conclusion reached by Brown *et al.* (1948). We have not observed any synergism between adrenaline and curare, like that described by Naess & Sirnes (1953). It would seem essential to eliminate decisively the possibility that this phenomenon was secondary to the large and prolonged vascular changes produced by adrenaline in rabbit muscle *in situ* (Girling, 1951).

Since adrenaline increases the amplitude of e.p.p.'s at junctions blocked by curare and magnesium (and possibly by fatigue), it does not seem likely that its action is due to a specific inhibition of curare. We have already shown that there is probably no sensitization of the end-plate to ACh. The most likely explanation is that there may be an increase in the amount of ACh released by each nerve impulse.

Changes in resting potential. Our results do not show any significant effect of adrenaline on the muscle fibre resting potential, confirming the negative observations made by Hutter & Loewenstein (1955) on frog muscle *in vitro*. Brown *et al.* (1950) found a small change in demarcation potential in cat muscle *in situ*, which they interpreted as an increase in resting potential. They correlated the absence of such an effect in frog muscle *in vitro* with the failure of adrenaline to augment the twitch tension of unfatigued frog muscle; however, adrenaline has been shown clearly to have a potentiating action on the unfatigued rat diaphragm *in vitro* (Goffart & Brown, 1947; Goffart, 1949, 1952).

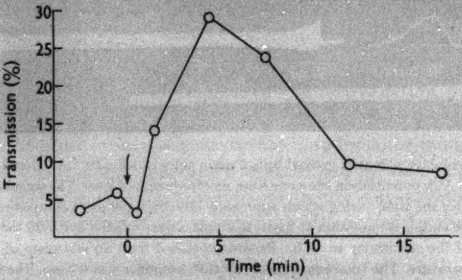

Fig. 9. Effect of adrenaline HCl (80 μg) applied at arrow, during a depression of neuromuscular propagation caused by the repetitive indirect stimulation of a diaphragm at room temperature. The intracellular electrode was not in an end-plate region. The resting potential was 60 mV and the frequency of stimulation 12·5/sec. Each point is the mean percentage rate of transmission over a period of 30 sec.

Conclusions

Adrenaline has a number of actions which influence neuromuscular propagation in several ways. It produces a reversal of presynaptic failure of conduction, and more ACh may be released by each nerve impulse under its influence. On the other hand, it also decreases the electrical excitability of the muscle fibre. Since these effects conflict with each other, the total result would depend upon which predominates. As a further complication, during continued repetitive activity the restoration of presynaptic conduction abolishes the protective effect of the failure, and hence may eventually predispose to a more complete post-synaptic block.

The over-all effect is usually probably beneficial as far as the contraction of the muscle is concerned. This is shown in Fig. 9 which illustrates the temporary improvement, under the influence of adrenaline, in the repetitive activity of a failing fibre recorded at a point distant from its end-plate. There is a striking change in the rate of firing; however, it should be noted that the effect is more transient than is usually the case *in vitro* when recording at an end-plate (Figs. 1, 2). This suggests that post-synaptic failure is setting a limit to the main presynaptic effect.

This multiplicity of, and mutual interference between, presynaptic and post-synaptic events may help to explain the variety of effects ascribed to adrenaline.

SUMMARY

1. We have studied the action of adrenaline on neuromuscular propagation in the rat diaphragm (*in vitro*) and gracilis (*in situ*).
2. In a majority of trials adrenaline relieved at least in some degree the intermittent presynaptic failure of conduction caused by tetanic stimulation of the motor nerve.
3. Adrenaline produced no detectable effect *in vitro* on potentials evoked artificially at end-plates with microjets of ACh, or on the amplitude of spontaneous miniature end-plate potentials.
4. Adrenaline (*in vitro* and *in situ*) increased the amplitude of e.p.p.'s in muscles paralysed by curare, excess Mg, or even fatigue. This suggests that it may cause more transmitter to be liberated at the neuromuscular junction.
5. Adrenaline *in vitro* tended to decrease the electrical excitability of muscle fibres stimulated at a low frequency. At high frequency it failed to change in any significant way the usual progressive increase in threshold.
6. Adrenaline regularly caused an acceleration of the spontaneous discharge of miniature e.p.p.'s. There was no appreciable action on the level of the resting membrane potential of muscle fibres.
7. The over-all action of adrenaline depends upon which effect predominates. Full restoration of presynaptic conduction may predispose to an eventual complete *post-synaptic* failure.

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SOME PROPERTIES OF FROG SKIN MECHANORECEPTORS

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The properties of single sensory units in the skin have been studied in the past using the single-fibre preparation. This has been achieved in the frog either by the recording of antidromic impulses evoked in a severed branch of skin nerve by stimulation of a nearby area of skin innervated by an adjacent nerve branch (Adrian, Cattell & Hoagland, 1931), or by isolation of single afferent fibres in a skin nerve (Maruhashi, Mizuguchi & Tasaki, 1952). An alternative method is to excite individual endings by sharply localized stimulation, and to record from the intact skin nerve. This has the advantages of saving much time in dissection, and of ensuring that measurements, such as velocity of conduction of afferent spikes, are made on the fibre in its normal situation in the nerve trunk, and not exposed to air. Thus using a fine shielded metal tip (diameter 20 μ) both for mechanical and electrical stimulation, it was possible to elicit single unit responses by either means of stimulation. Owing to the simple nature of the method many observations could be made on one preparation. Preliminary accounts of parts of this work have already been presented (Catton, 1957*a*, *b*).

The sensory innervation of frog skin may be briefly described as follows. Myelinated and non-myelinated axons entering the subepidermal layers form a deep and a superficial plexus. From these plexuses arise axons which branch into fine non-myelinated fibres, some ending in the subepidermal layers, some in association with the skin glands and blood vessels and many penetrating and ending freely in the epidermis (Ecker & Wiedersheim, 1904). Only one kind of anatomically differentiated ending has been described, the so-called 'tactile corpuscle' (Merkel, 1880), having a limited distribution. Eberth & Bunge (1893) described 'endzellen', lying close under the epidermis, from which some of the epidermal endings were derived; these do not appear to have been described by subsequent authors. The general picture is one of a closely interwoven network of naked axons, derived by profuse branching of the myelinated sensory axons, and showing extensive overlapping of areas innervated by single afferent fibres. The dermal endings have been described in more detail using modern techniques (Whitear, 1955).

METHODS

Portions of calf skin of *Rana temporaria*, with attached nerve branch (ramus cutaneus cruralis medius), were mounted in a Perspex chamber. The skin was laid on a silver plate serving as earthed indifferent electrode, and the nerve was laid across two silver wire electrodes connected to the amplifier. The whole preparation was covered with liquid paraffin, and was found to remain responsive for several hours. The chamber was mounted in a mechanical stage on a microscope stand, so that it could be moved in the horizontal plane by small measured amounts. The $20\,\mu$ shielded electrode, forming part of the stimulator unit (Fig. 1), was moved only in the vertical plane. The stimulator unit was mounted on the focusing rack, the microscope body being discarded, so that the normal focusing controls were available for setting the initial level of the electrode tip. The unit shown in Fig. 1, using solenoid and armature drive, was the one used in later experiments; it had the advantage that it could be driven by one pulse of a two-pulse electronic stimulator, with the interposition of a single-stage power amplifier to provide the solenoid current. Since the solenoid was some distance away from the pick-up leads, very little artifact due to the current pulse appeared on the oscillograph trace. The rise time of the mechanical pulse was nearly constant at 1·5 msec so that the slope of rise varied directly with amplitude. An earlier

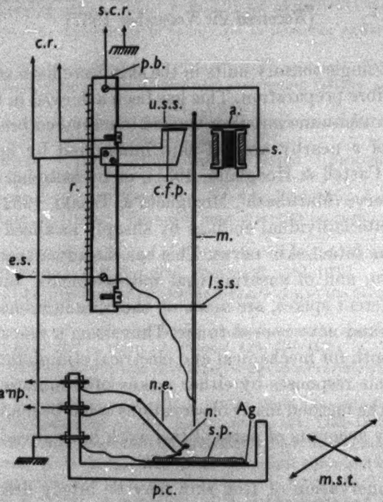

Fig. 1. Diagram of apparatus for skin stimulation. *c.r.*, capacitance recorder; *s.c.r.*, solenoid current supply; *p.b.*, Perspex bar; *u.s.s.*, upper support strip; *a*, armature; *s*, solenoid; *c.f.p.*, capacitor fixed plate; *m.*, micro-electrode; *l.s.s.*, lower support strip; *n.*, nerve; *s.p.*, skin preparation; *Ag*, silver plate; *p.c.*, Perspex chamber; *n.e.*, nerve electrodes; *r.*, rack; *amp.*, leads to amplifier; *e.s.*, lead from stimulator; *m.s.t.*, mechanical stage traverse.

stimulator used pneumatic transmission; the amplitude was nearly constant, but a wide range of slopes could be obtained. With either arrangement the maximum displacement obtainable was about 50 μ, but movements of the order of 5 μ were usually sufficient to evoke responses from single units. The absolute amplitudes of the movements were not recorded, but the movements were monitored by a simple capacitor device attached to one of the electrode support strips. The capacity change was amplified and passed to the second beam of the oscillograph, the nerve responses appearing on the first beam. Electrical pulse stimuli, derived from the second pulse output of the electronic stimulator, were applied to the electrode via a radio-link isolating unit, to minimize the artifact. The latencies of the mechanically evoked responses were measured from the commencement of the mechanical displacement; for the electrically evoked responses, latencies were measured from the electrical artifact. Conduction velocities in main sensory axons were estimated from observed conduction times when stimulation was applied to intradermal nerve branches, and by measuring the length of nerve between these points and the first recording electrode.

RESULTS

Impulse discharges evoked by mechanical stimulation

The discharges evoked by point stimulation could be placed in four main categories: (*a*) large, fast-conducted spikes of amplitude 350–450 μV, conduction velocity 20–30 m/sec; (*b*) medium size and conduction velocity, 200–300 μV and 10–15 m/sec respectively; (*c*) small size and velocity, 100–150 μV and 5–10 m/sec respectively, but still in the myelinated range; (*d*) small, slowly conducted spikes, having the low broad-contour profile characteristic of spikes in non-myelinated fibres, amplitude <100 μV, velocity 0·1–0·3 m/sec. Further properties were as follows.

Type (a) discharge. These were evoked either by gentle stroking of the outer skin surface, by rapid vertical displacements applied from the inside or outside, or by slower displacements when the point was pressing on the inside surface of the epidermis (Fig. 2i, iii, iv). Stroking of the outer surface evoked these spikes almost exclusively, but they could only be obtained from numerous but discrete limited areas, each about 50 μ square. Sections of skin from such areas were stained by methylene blue and silver methods, but heavy pigmentation made interpretation difficult, and no evidence of specialized endings was found. These large spikes were very rarely discharged more than once per stimulus (Figs. 2iii, iv; 3ii). Under rapid repetitive stimulation they appeared as very brief trains at 200/sec (Fig. 5iii) and more usually followed the stimuli only at frequencies of up to about 3/sec. The maximum following rate was dependent on stimulus strength: thus when a spike failed to follow as the frequency was raised it could be made to follow again by increasing the stimulus strength; when it became fatigued at the new frequency, once again an increase in the stimulus would cause it to follow. A similar phenomenon was described by Keidel (1955) but probably for fibres of types (*b*) or (*c*) below. This threshold-raising effect of repetitive stimulation is illustrated in Fig. 6 for four different type (*b*) responses. The latency of the type (*a*) spikes varied with rate of displacement of the stimulating tip, being as long as 50 msec

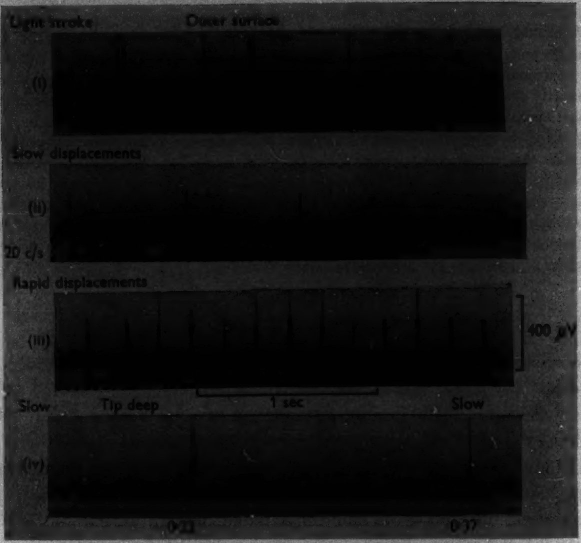

Fig. 2. Sensory ending discharges in skin nerve in response to mechanical stimuli. (i) Type (*a*) discharges evoked by light stroking of outer surface; (ii) Type (*b*) discharges evoked by slow displacements applied to inner surface; (iii) Types (*a*) and (*b*) discharges evoked by rapid displacements at same site as (ii); (iv) Type (*d*) discharges evoked by steady pressure of tip against inside of epidermis, with superimposed slow displacements, signalled by lower trace, evoking type (*a*) discharges. Time marker records (ii) and (iii), 20 c/s.

for very slow, near-threshold displacements, shortening to 5–10 msec for the most rapid.

Type (*b*) *discharge.* Spikes in the medium range could be evoked by vertical displacements applied to the inner or outer surfaces, although more readily from the inner. They were obtained generally at lower displacement rates than were needed to produce type (*a*) discharges (Fig. 2ii, iii) and tended to fire repetitively at higher rates (Fig. 2iii). When stimuli were applied from the inside, as the point was progressively sunk deeper the type (*b*) spikes were obtained first, type (*a*) later, and when the point was resting on the inside of the epidermis only the type (*a*) discharge could be evoked (Fig. 2iv). Repetitive discharges of up to 15 spikes at frequencies of 60/sec could be elicited from fresh preparations when large, briefly maintained displacements were used. Under repetitive mechanical stimulation the type (*b*) spikes followed at frequencies of 300/sec for very brief periods (Fig. 5ii), at 100/sec for longer

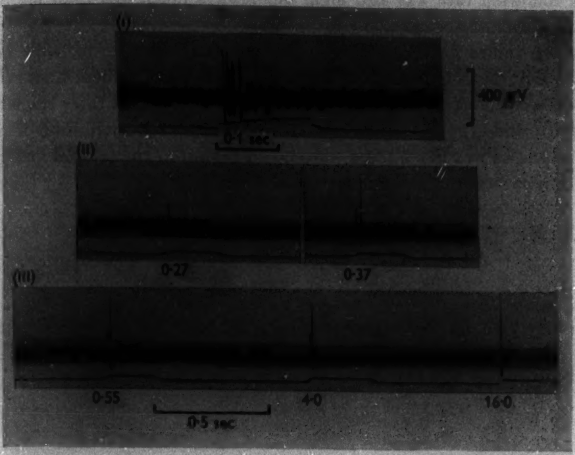

Fig. 3. (i) Train of discharges (upper trace) initiated by a single displacement (lower trace lasting 0·12 sec; note delayed small fibre discharges. (ii, iii) Effect of increasing rate of displacement on latencies of medium and large fibre discharges; rate of displacement shown below record (see text).

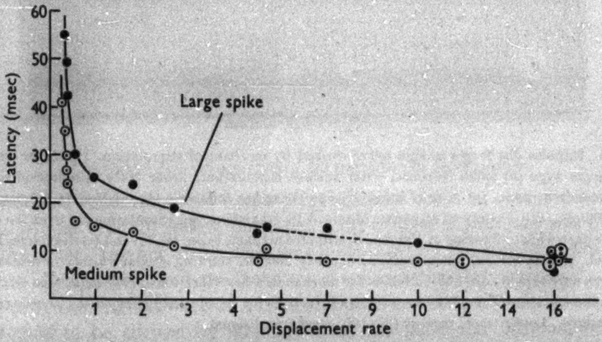

Fig. 4. Latency values for a large (●) and a medium (⊙) spike response plotted against displacement rate (see Fig. 3). The graph has been compressed on the horizontal axis by omission of points at 6 and 8; note altered abscissa scale.

periods (Fig. 5iii) and at 20/sec for several seconds (Fig. 5v, vi). There was a wide variation in the maximum following frequency for different endings. Generally if the following rate of the unfatigued ending was high, then the steady following rate was also high, but always much less than for the unfatigued state.

Latency values for medium spikes ranged from 40 msec for slow rates of displacement down to 5–10 msec for rapid rates. At all except high rates the latencies were shorter than for type (*a*) spikes, but at high rates the latencies for type (*a*) and type (*b*) became equal (Figs. 3ii, 4). The distribution of type (*b*) spike responses was much more widespread than for type (*a*) and localization of the former was correspondingly more difficult.

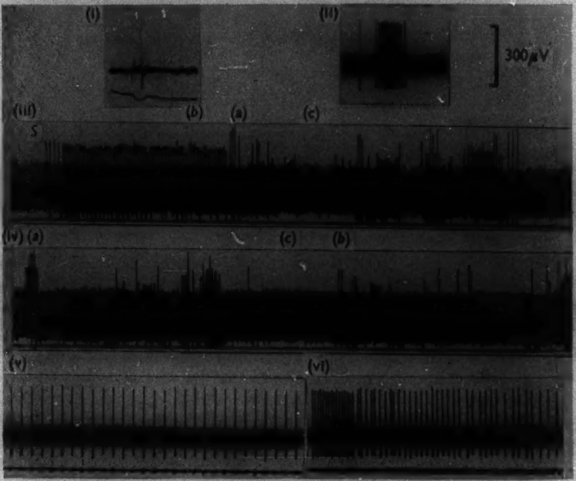

Fig. 5. Impulse discharges in skin nerve evoked by mechanical stimulation. (i) Upper trace, a single type (*a*) spike response, outer surface stimulation; lower trace, mechanical signal; latency 5 msec. (ii) A brief burst of type (*b*) spikes following stimulation at frequency of 300/sec. (iii) Variety of responses obtained by traversing the stimulating tip over the outer surface whilst vibrating at 100/sec; *S*, stimulus artifact, types (*a*), (*b*) and (*c*) discharges labelled. (iv) As for (iii) but vibration frequency 200/sec, showing similar variety of discharges, but only type (*c*) are able to follow for more than a few vibrations. (v) Single type (*b*) spikes following stimuli at a frequency of 20/sec. (vi) As for (v) but frequency 60/sec, showing rapid fatigue. Lower trace, records (iii)–(vi), mechanical signal.

Type (*c*) *discharges*. These had the characteristics of spikes propagated in small myelinated axons. They were most readily evoked by rapid repetitive stimulation and followed at frequencies of 200/sec or more, with less adaptation than was shown for type (*a*) and (*b*) (Fig. 5iv). Their distribution was widespread, as for type (*b*). Spikes in the same range of sizes were produced by various forms of chemical stimulation, by dilute NaCl and HNO_3, as also by ACh.

Type (*d*) *discharges*. These had the characteristics of spikes propagated in non-myelinated axons. Fresh preparations showed irregular discharges which persisted in the absence of stimulation for periods of up to 10 min. They could then be readily elicited by pressing the electrode point firmly against the inside of the epidermis (Fig. 2iv). Occasionally a short burst would follow a single displacement stimulus after a long latency (Fig. 3i). Type (*d*) spike responses showed little or no adaptation to maintained stimulation. Their distribution was widespread, and latency values ranged from 40 to 100 msec.

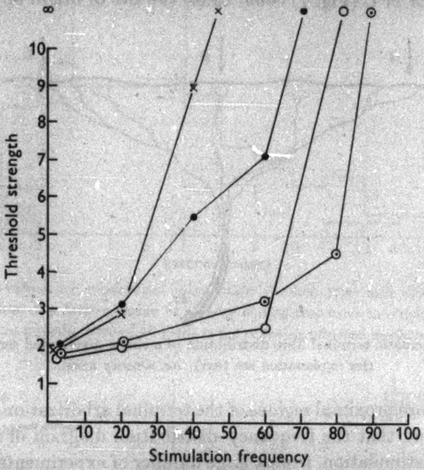

Fig. 6. Variation of threshold with frequency of stimulation for some medium spike responses.

Localized discharges evoked by mechanical stimulation

Localized spike responses to the point stimulus could be obtained both for type (*a*) and type (*b*) spikes, but much more critical adjustment was needed for the latter. In view of the wide area innervated by the numerous branches of one afferent axon and the fact that these areas overlap extensively, as is shown by the original work of Adrian and others, such localized responses are presumed to be elicited by stimulation of one particular terminal branch. This view is supported by various observations. Thus, when the tip, whilst undergoing mechanical movements at a steady frequency was traversed through very small distances in the vicinity of one such isolated response, regular rapid shifts of latency occurred for the identical impulse as picked up in the sensory axon. A position could be found where the latency had a minimum value, all movements from this site causing an increase in latency. A possible interpretation of this result, related to the terminal branches of a single sensory axon, is as follows. Stimulation at *B* (Fig. 7) would result in a shorter latency of response than stimulation at *A* or *C*, owing to the shorter length of nerve ending underlying *B*. This variability of latency in relation to the precise position of the stimulating tip constitutes one variable factor in mechanical-response–latency measurements, in addition to the well known 'latency-shortening' effect of increased stimulus strength. Conceivably the two might have a common origin, in that increased displacements applied at points such as *A* or *C* (Fig. 7) could cause the site of origin of the impulse to be shifted to more proximal regions of the terminal arborization. It is not then surprising to find that the frequency–distribution diagram of latency values for mechanical stimulation, from a large number of experiments, shows a wide scatter (Fig. 8). On the same diagram are plotted the results from all electrical stimulation experiments (see below), and it is seen that the distribution of latencies to mechanical and electrical stimulation are generally similar, although discharges in response to electrical stimulation are evoked more commonly than those to mechanical stimulation in the ranges 1–4 and 7–13 msec. The former may represent a proportion of cases where nerve branches were directly stimulated. the latter may include non-mechanoreceptor responses.

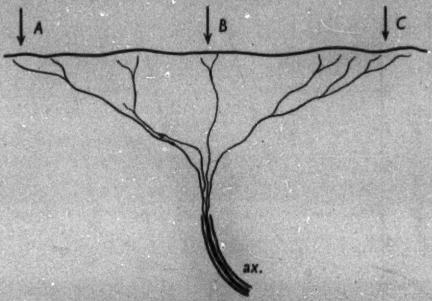

Fig. 7. Schematic terminal fibre distribution of a single myelinated sensory axon (for explanation see text). *ax.* sensory axon.

Spike discharges evoked by localized electrical stimulation

When an electrical pulse of duration 0·2 msec or longer was applied to the skin via the 20 μ shielded electrode, a pattern of spike discharges of varying latencies was recorded from the cutaneous nerve. It was found that many of these responses, particularly those of longer latency, were rapidly fatigued by such stimulation, and it was necessary to use frequencies not higher than 1 or

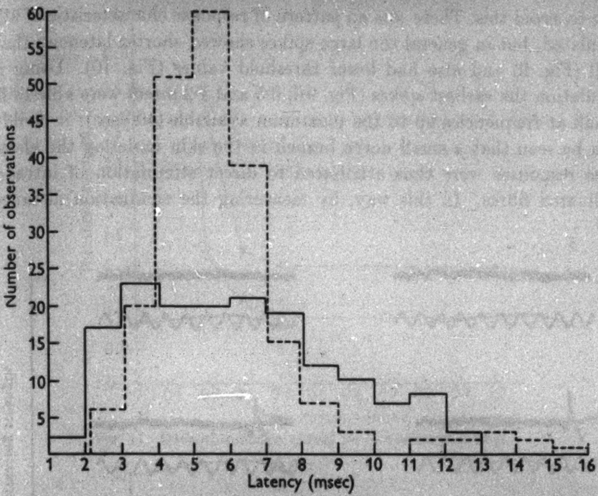

Fig. 8. Latency values for mechanical stimulation (broken line) and electrical stimulation (continuous line) of a large number of sensory ending responses to outside surface stimulation. The values include those of all three types of fast-conducted mechanoreceptor response described.

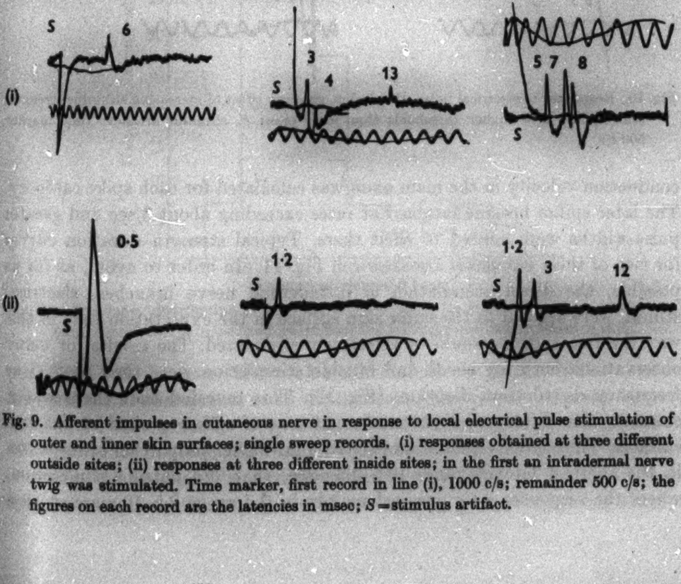

Fig. 9. Afferent impulses in cutaneous nerve in response to local electrical pulse stimulation of outer and inner skin surfaces; single sweep records. (i) responses obtained at three different outside sites; (ii) responses at three different inside sites; in the first an intradermal nerve twig was stimulated. Time marker, first record in line (i), 1000 c/s; remainder 500 c/s; the figures on each record are the latencies in msec; S = stimulus artifact.

2/sec to avoid this. There was no pattern of response characteristic of all sites stimulated, but in general the large spikes showed shorter latencies than the small (Fig. 9) and also had lower threshold values (Fig. 10). Using inside stimulation the earliest spikes (Fig. 9ii, 0·5 and 1·2 msec) were able to follow stimuli at frequencies up to the maximum available (400/sec); it could then often be seen that a small nerve branch in the skin underlay the electrode. These responses were thus attributed to direct stimulation of intradermal myelinated fibres. In this way, by measuring the conduction distance the conduction velocity in the main axon was calculated for each spike category. The later spikes became fatigued at rates exceeding about 3/sec and greater pulse-widths were needed to elicit them. Typical strength–duration curves for two of these responses are shown in Fig. 11. In order to avoid, as far as possible, the direct stimulation of intradermal nerve branches, electrical stimuli were applied to the outer skin surface in the expectation that in this way the free endings would be selectively stimulated. The results for many observations both for inside and outside stimulation were then plotted as frequency–distribution diagrams (Fig. 12). This revealed that there was a characteristic difference between the patterns of spikes evoked at the two surfaces. Thus inside stimulation elicited a far higher proportion of responses in the shortest latency range (0·5–2·5 msec) than did outside stimulation, where the responses were grouped mainly in the range 2·5–10 msec, with a

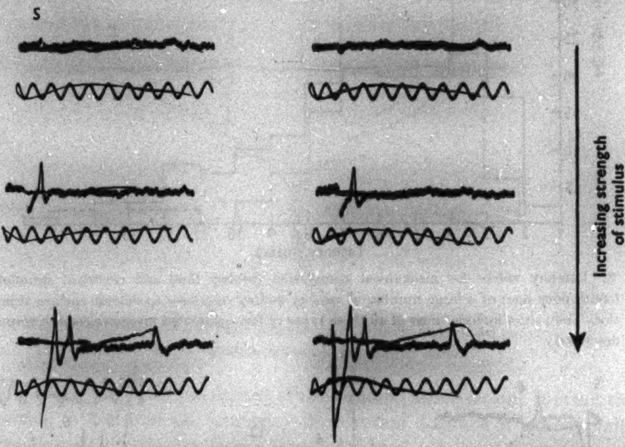

Fig. 10. Responses to electrical pulse stimulation, showing effect of increasing stimulus strength; later spikes have higher thresholds than early ones; *S*, stimulus artifact; time marker, 500 c/s.

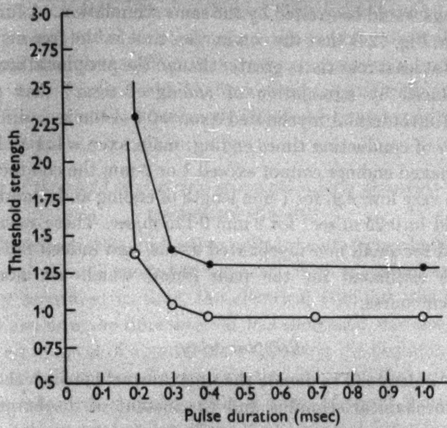

Fig. 11. Strength-duration curves for two sensory endings.

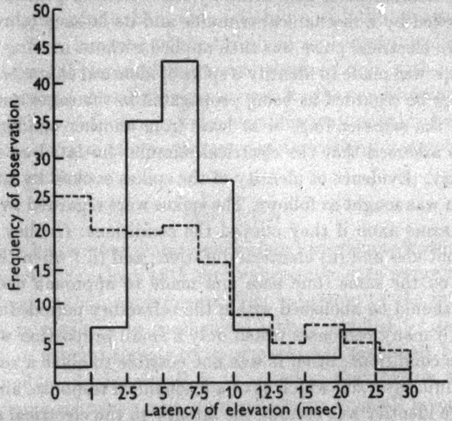

Fig. 12. Frequency distribution of observed latency values for sensory responses to electrical pulse stimulation of outer (continuous line) and inner (broken line) surfaces of skin.

peak from 5 to 7·5 msec. This peak would include the peak of latency values for mechanical stimulation in all experiments (Fig. 8) and the mean latency value of 5·1 msec given by Gray & Malcolm (1951) for mechanical stimulation of the dorsal skin–nerve preparation. This seems to suggest that the majority of the spikes produced by outside electrical stimulation were derived from mechanoreceptors, although it would be expected that thermal and chemoreceptor endings would be excited by the same stimulation. A further possible deduction from Fig. 12 is that the conduction time in the free nerve endings is of the order of at least four times greater than in the peripheral axon. (Latency of spike produced by stimulation of ending = 5 msec; that produced by stimulation of intradermal myelinated axon = 0·5–1·0 msec; difference = 4·0–4·5 msec; ratio of conduction times ending: main axon = 4:1–9:1). Since the length of the naked endings cannot exceed 1 or 2 mm the conduction velocity would then be very low, e.g. for 1 mm length of ending and conduction time of 4 msec it would be 0·25 m/sec; for 2 mm 0·125 m/sec. These values are in the range expected for small non-myelinated axons, and indeed fall in the range (0·1–0·3 m/sec) estimated for the pain fibres, which are non-myelinated throughout their course.

Receptor delay

This term is understood to denote the time interval between the commencement of the mechanical stimulus and the instant of discharge of the first impulse at the mechanoreceptor ending. The technique employed seemed to permit the measurement of this delay by the following procedure. A single spike was evoked by a mechanical stimulus and its latency (threshold value) was noted. An electrical pulse was then applied without moving the electrode and an attempt was made to identify a spike of identical characteristics, which could therefore be regarded as being propagated in the same axon and hence derived from the same ending, or at least from another ending of the same axon. It was assumed that the electrical stimulus initiated an impulse with negligible delay. Evidence of identity of the spikes evoked by the two modes of stimulation was sought as follows. The spikes were regarded as being propagated in the same axon if they obeyed the conditions: (i) they should have identical amplitudes and (ii) identical duration; and (iii) when the two spikes were evoked on the same time base and made to approach each other, the second spike should be abolished within the refractory period due to the first (1–3 msec). Of many responses tested only a small proportion were found to fulfil all these conditions; often it was not possible to elicit a response to an electrical stimulus at a site which gave a mechanical response, and vice versa. In cases where identity was certain, the latency to the electrical stimulus was in general shorter than to the mechanical stimulus, although in some cases the electrical latency was longer. The time differences observed covered a fairly wide range. Out of 70 cases the values of receptor delay (mechanical latency minus electrical latency) ranged from 0·5 to 4·5 msec, 55 being in the range 1–3 msec and 46 of these in the range 1·5–3 msec. No distinction could be made between receptor delays for types (*a*), (*b*) and (*c*) responses, all giving values falling within the full range. A reasonable deduction would be that the receptor delay at these endings is similar for all fast-conducted mechanical responses and has a value close to 2 msec. The value would in any case be expected to be small and of this order, since the plots for all results of electrical and mechanical stimulation (Fig. 8) show no evidence of a large relative displacement in time. Further evidence for a change in excitability of the endings at a time interval of about 2 msec was afforded by the experiments reported in the next section.

Lowering of threshold to electrical stimulation by a preceding subliminal mechanical stimulus

Subliminal mechanical stimuli were applied at a site from which both electrical and mechanical stimuli evoked a response. With electrical and mechanical pulses widely separated in time, the electrical stimulus was set just below threshold. When now the time interval was shortened, the electrical response appeared at an interval of about 2 msec following the beginning of the mechanical pulse, and disappeared again at a time interval of about 6 msec. Thus, following a mechanical stimulus, after a delay of about 2 msec, the excitability of the ending rises and remains above normal for a further period of about 4 msec. The effect was of a marginal nature and it was not possible to plot the time course of the change in excitability with the apparatus available. The converse effect, lowering of the threshold to a mechanical stimulus by a preceding subliminal electrical pulse, was obtained only with inside siting of the electrode, using outward-flowing current for the conditioning pulse. These excitability changes following a pulse stimulus may possibly be presumed to indicate the time course of a membrane depolarization ('generator potential') at the sensory endings.

DISCUSSION

Since we do not know what sensations the frog experiences from stimulation of its skin, we work by analogy with such comparable data as are accepted from human and mammalian experiments. The behaviour of the intact frog confirms that it is sensitive to mechanical stimuli applied to its skin and its responses are in general similar to those evoked in mammals by comparable types of stimulus. In mammals it is accepted that tactile sensations are mediated in large sensory axons, pain sensations in small myelinated and in non-myelinated axons. The sensory discharges are further characterized by differences in rate of adaptation to maintained stimulation.

The following sensory functions may be tentatively assigned to the four categories of mechanoreceptor described:

Type (*a*), fast-conducted, fast-adapting discharges from touch receptors probably sited mainly in the epidermis. They appear to correspond to the type A_1 endings of Fessard & Segers (1943), to the tactile endings of Maruhashi *et al.* (1952) and to the touch endings studied by Loewenstein (1956), all on frog skin.

Type (*b*), slower conduction and slower adaptation than for type (*a*); probably sited mainly in the subepidermal layers. They seem to correspond to the type A_2 endings (slowly-adapting touch receptors) of Fessard & Segers, and to the 'stretch' endings studied by Loewenstein. The effective mechanical deformation which initiates an impulse is in all cases probably a stretching of the naked fibre termination. Thus, whether we call type (*b*) spikes slowly adapting touch, pressure or stretch responses depends essentially on the mode of application of the mechanical stimulus. In the present experiments the skin was not grossly stretched, but the type (*b*) spikes have the same characteristics as those evoked by stretch in Loewenstein's experiments. The microanatomy of the subepidermal endings has been described by Whitear (1955).

Type (*c*) spikes show yet slower conduction and slower adaptation than type (*b*). Since they are particularly readily evoked by rapid repetitive stimulation, they seem well fitted to subserve the role of vibration-receptors, if vibration sense is to be regarded as a separate modality. It has been shown that the minimum movement required to evoke a sensation of vibration at the finger tip in man is about $0{\cdot}02\,\mu$ at 400 c/s (Wilska, 1954). There is the possibility that the type (*c*) discharges may originate primarily from chemo- or thermoreceptors stimulated anomalously in this way, since they fall in the same range of spike size and it is known that the converse effect is possible, i.e. that mechano-receptor responses can be evoked by thermal stimulation (Hensel & Zotterman, 1951; Dodt, 1955).

Type (*d*) discharges clearly represent slowly-conducted pain.

A comparison of the properties of types (*a*) (*b*) and (*d*) discharges with the categories A_1, A_2 and 'pain' described by Fessard & Segers is given in Table 1. Clearly with the present method the spike amplitudes are about ten times greater, the spike duration of the larger fibres much shorter, and the conduction velocity of the non-myelinated fibres much lower. These results are much more in accord with the usual properties of frog nerve fibres and may reflect a more nearly normal preparation.

Electro-physiological investigations of the sensory responses of mammalian skin have indicated that in a general way it is possible to correlate specific modes of sensation with characteristic discharges in corresponding types of afferent fibre, grouped according to diameter (and hence according to spike amplitude and conduction velocity). Such findings have not been widely accepted by many anatomists who have closely studied the morphology of mammalian cutaneous sensory endings, and little correlation appears to exist at present between the physiological and anatomical schools of investigation. Thus modern anatomical studies (e.g. Weddell, Palmer & Pallie, 1955) have led to the conclusion that all nerve fibres entering mammalian skin terminate in arborizations of fine ($<1\,\mu$) naked exoplasmic filaments which probably end freely. This is identical with the picture in frog skin. So far has the anatomical school deviated from acceptance of the doctrine of 'specific nerve energies', that Sinclair (1955) has stated the view that cutaneous sensation is mediated by a spatially and temporally dispersed pattern of impulses leaving the skin. The physical characteristics of this pattern are to be the determining factors in the experience of sensory quality, with the conclusion that there are no histological grounds on which to erect theories of cutaneous sensibility based on the existence of four primary modalities, touch, warmth, cold and pain, operating within the 'law of specific nerve energies'. Further, that there is no evidence for the existence of specific nerve endings subserving these modalities. Certainly recent work (e.g. Lele & Weddell, 1956) has shown that the cornea, possessing only free nerve endings, subserves touch and temperature, as well as pain sensation. In general, then, the ability of a system of free nerve endings to originate impulse discharges in sensory axons related to specific modalities of sensation, although difficult to explain in the context of our present knowledge of the mode of origin of the sensory impulse, is a fairly well substantiated process. On the other hand, views such as those expressed above must be regarded as extreme, in that they leave no role for the highly specialized encapsulated endings dispersed so widely in mammalian skin.

TABLE 1. Summary of characteristics of frog skin mechanoreceptors

	Fast-adapting touch		Slow-adapting touch or stretch			
	A_1*	Type (*a*)†	A^2*	Type (*b*)†	'Pain'*	Type (*d*)†
Spike size (μV)	30–50	350–450	10–20	200–300	1–5	100
Spike duration (msec)	2·3	1	3·5	1	7–18	5–15
Adaptation rate	Quasi-instantaneous	Instantaneous	'a few seconds'	0·5 sec	'Several minutes'	Of the order of minutes
Conduction velocity (m/sec)	18–20	20	11–14	10–15	'A few m/sec'	0·1–0·3
Minimum displacement rate to elicit	High	High	Moderate	Moderate	—	—
Rate of following repetitive stimulation	Above 75/sec	300/sec unfatigued 5/sec fatigued	Below 50/sec	300/sec unfatigued 20/sec fatigued	—	—

* Responses described by Fessard & Segers (1943); † Responses described in present work.

As regards the present work it has been shown that at least four types of mechanoreceptor are found in frog skin, having characteristic properties. The question of choice of terminology has arisen in designating the recorded impulses. Thus in earlier work it was customary to use the terms 'fast' and 'slow' to designate the large tactile spikes and the very small and broad contour spikes of pain receptors respectively. Since spike size and conduction velocity in myelinated fibres are closely proportionate quantities, in general either term may be used for descriptive purposes in a constant experimental situation.

But what is generally observed in the first instance is spike size, and the estimate of conduction velocity could only be approximate with the method used. Thus the responses were classified according to size and such estimates of conduction velocity as could be made showed that there was a general proportionality between spike size and velocity.

The variation reported here of latency with rate of mechanical displacement covers a wider range than has been recorded previously. This may be due to the wide range over which the rate of displacement could be varied in these experiments. We may briefly speculate on the factors which may contribute to the latency-shortening effect of increased rates of displacement. The following sources of delay must be considered: (*a*) a short delay, due to the transmission time of the wave of mechanical displacement through the tissues; (*b*) the time taken for the 'generator potential' at the ending to reach the critical amplitude for the firing of an impulse; and (*c*) variable delay due to excitation occurring progressively at points nearer the main axons as the stimulus strength rises. Of these (*a*) is not known, but is likely to be small and constant; (*b*) is also not known but is likely to be of the order of 1 msec or so (cf. results of Gray & Sato (1953) on Pacinian corpuscle of cat's mesentery), and variable over a short range; (*c*) seems most likely to contribute most of the variation observed, but can only do so within the limits of the conduction time along the naked sensory fibre termination, which is of the order of 4 msec. The sum of all these delays (perhaps 10 msec) thus falls far short of the longest latencies (40 msec or more) measured experimentally, and no explanation for these long latencies is immediately evident.

The method used for measurement of receptor delay is based on the three assumptions: (*x*) that an electrical stimulus initiates an impulse at the ending with negligible delay, (*y*) that the mechanical stimulus evokes an impulse discharged in the same axon, and (*z*) that each type of stimulus initiates the discharge at the same region of the extended sensory termination. Of these, (*z*) is possibly the least justifiable. It seems likely that, as described above, the point of excitation to a considerable extent determines the value of latency measured, according to the length of naked fibre termination which must be traversed before the impulse reaches the beginning of the main axon. Since the conduction time in these naked terminations is long compared to that in the main axon, marked errors in latency estimation will be associated with shifting of the effective point of excitation either along the length of one termination, or from the tip of one to the tip of another of shorter length. Taking into account these uncertainties which perhaps account for the wide range in estimated receptor delays (1–10 msec), it is surprising to find that nearly 80% of values measured fall in the range of 1–3 msec. This range includes the values from 1·5 to 3 msec obtained for receptor delay at the Pacinian corpuscle (Gray & Malcolm, 1950; Gray & Sato, 1953).

It is difficult to account for the finding that the responses to electrical pulse stimuli fatigue much more readily than do those to mechanical stimuli. Thus for mechanical stimulation many of the smaller responses (type *c*) follow at frequencies of 200/sec or more; yet none of the electrical responses which could be excluded as direct nerve responses were capable of following at frequencies higher than about 3/sec. Used in this sense, fatigue might be regarded as an index of rate of adaptation or accommodation, being measured in terms of rise of the threshold value. Thus electrical stimulation seems to bring about a very marked accommodation, or alternatively is effective at a region where accommodation is more rapid than for mechanical stimulation.

There still remains the problem of differentiation of sensory function among a population of sensory endings presenting apparently homogeneous morphological characters. Loewenstein (1956) has suggested a possible basis for such functional differentiation in the case of frog skin. He found that the application of constant stretch to a skin preparation altered the characteristics of the normally fast-adapting touch receptors so that they behaved in the same way as the more slowly adapting 'stretch' receptors. According to Loewenstein the fast-adapting endings are in a state of relaxation, with their membranes partially folded, whereas the 'stretch' endings are normally extended, with no folding of their membranes. The application of constant stretch causes unfolding of the membranes of the fast-adapting receptors; in this 'biased' condition they now become slow-adapting. A similar suggestion has been made for the specialized stretch receptor cells of certain Crustacea (Eyzaguirre & Kuffler, 1955). For the slow-adapting RM_1 receptor less slippage is possible between the dendrite terminals and surrounding tissue than for the RM_2 type (fast-adapting), so that the RM_1 type could be under some degree of tension before application of the mechanical stimulus.

SUMMARY

1. A method is described for applying to frog skin sharply localized mechanical or electrical stimuli, separately or in combination. Single sensory unit discharges could be recorded by the method.

2. By mechanical stimulation four types of spike responses could be recorded from the cutaneous nerve; the characteristics and probable functional correlations of these responses are described.

3. Electrical pulse stimulation evoked patterns of spike responses of a wide range of latency values; stimulation of the inner surface produced a much higher proportion of short-latency responses than was found for outside stimulation. It could be inferred from the results of electrical stimulation that conduction of the sensory impulse in the fine naked axon terminations was much slower than in the main myelinated axon, and an approximate value for

conduction velocity in these endings would be 0·25 m/sec. The responses to electrical stimulation generally fatigued at much lower frequencies than did those to mechanical stimulation of the same area.

4. Receptor delay for these endings was estimated by making independent measurements of the spike responses of the same ending to electrical and to mechanical stimuli. Most values fell in the range 1·5–3 msec.

5. Following a subliminal mechanical stimulus the excitability of the endings begins to be enhanced at a time of 1·5–3 msec after the application of such a stimulus. The excitability remains above normal for a further period of 3–6 msec. This may represent the approximate time course of the 'generator potential'.

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RENAL FUNCTION IN THE FOETAL AND NEW-BORN GUINEA-PIG

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Renal function before birth has been studied in comparatively few species, and observations on the lamb, rat and human infant have contributed the greater part of our knowledge. When Jacqué (1905–06) was investigating the origin of amniotic and allantoic fluids in the sheep, he determined the osmolar concentrations of these fluids and compared them with those of the foetal urine and plasma. The foetal urine was hypotonic (average 138 m-osmole/l.) and the changes in the osmolar and chloride concentrations of allantoic fluid suggested to Jacqué that it was largely the product of the foetal kidney. This theory was supported by Alexander, Nixon, Widdas & Wohlzogen (1955*a*, *b*), who described changes in the composition of the foetal fluids and urine of the sheep during gestation. It has also been applied to rabbits (Davies & Routh, 1957).

Wells (1946) ligated the urogenital papilla of rat foetuses shortly before term and measured the volume of urine found in the bladder 2 hr later. If the volume found in the bladders of untouched litter-mates was deducted, the figures indicated that urine accumulated at the rate of 0·015 ml./g body wt./hr. Urines collected in this way over a 24 hr period (Daly, Wells & Evans, 1947) contained an average concentration of 195 mg urea N/100 ml. and, in one observation, a creatinine concentration of 42 mg/100 ml. Although these urines probably had a higher osmolar concentration than plasma, they had a lower concentration of urea and chloride than the maternal urine formed in the last 2 hr of the experiment.

McCance & Widdowson (1953) summarized the knowledge then available about urine formed by the human foetus and added a detailed study of twelve urines collected at delivery. These urines were invariably hypotonic, with an average concentration of 137 m-osmole/l., and the urea and creatinine urine: plasma ratios seemed to indicate that the glomerular filtrates had passed rapidly through the tubules without much concentration having taken place in the distal portions. Hanon, Coquoin-Carnot & Pignard (1955, 1957*a*, *b*) have more recently analysed twelve more samples of human urine formed *in utero*, and their results are in general agreement, particularly as regards tonicity. They consider, however, that the products of the kidney have no significant excretory function before birth.

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More data are at hand about the development of renal function after than before birth in experimental animals and man (McCance, 1950; Heller, 1951; Smith, 1951; McCance & Widdowson, 1954*a*). Evidence cited in these writings and supported by more recently published material (McCance & Widdowson, 1954*b*, 1955; McCance, Naylor & Widdowson, 1954; Falk, 1955) indicates that the kidney of the new-born mammal has a smaller concentrating capacity than that of an adult, a much lower rate of glomerular filtration per unit of body weight or of surface area, a smaller and usually a delayed excretion of water after a standard dose, and lower sodium and chloride clearances. These functions have been shown to mature at characteristically different rates and there are known to be considerable differences in this and other respects between rat, dog and man.

In the present study some observations have been made of the renal function of foetal guinea-pigs and these have been compared with similar observations in new-born and older animals.

METHODS

Guinea-pigs of the usual laboratory type were employed. The age of foetuses was in most instances known to within one week from the time of exposure of a group of does to a buck. The period of gestation in this species varies from 65 to 72 days depending upon whether conception occurs in a virgin or lactating doe, for in the latter implantation of the fertilized ovum may be delayed.

The urethral orifice of the female guinea-pig is accessible and a catheter of appropriate size can readily be introduced. In foetal and new-born animals a short length of polythene tubing was used for this purpose. At the beginning and end of each period over which urine was being collected the bladder was filled two or three times with air, and gentle supra-pubic pressure was applied. This enabled the bladder to be emptied. Blood was drawn into a heparinized syringe at the end of the period over which urine had been collected, in foetal animals from the umbilical vein, in the new-born from the bifurcation of the inferior vena cava or the aorta, and in adults from the heart. Urine was collected from the pregnant does by catheterizing them at the beginning of the experiment.

Foetal urine was collected in the following manner. The gravid doe was restrained on a small animal board with the hind legs crossed to give a semi-lateral presentation of the uppermost side. After infiltrating the abdominal wall with 2% procaine, a horizontal incision 6–7 cm in length was made across the exposed flank and abdomen 2–3 cm below the superior limit of the uterus as determined by palpation. The incision was carried through the subcutaneous tissue throughout its length and opened into the peritoneal cavity at its lateral extremity. The uterus was then attached to the upper edge of the peritoneal incision by a running suture which was continued medially as the incision into the peritoneal cavity was extended. The suture was then extended around the lower border of the incision to make a window of uterine wall sealed from the body cavity. The uterus was opened, and the hind quarters of the presenting foetus delivered. If necessary a version was performed. If the foetus was a female it was catheterized and the urine present in the bladder collected into a weighed tube. If the foetus was a male it was delivered and the second foetus, if female, was catheterized. The term urine 'found' has been applied to the urine found in and removed from the bladder on passing the catheter, and urine 'formed' to the urine which collected in the bladder during the subsequent period of observation. After the urine 'found' had been collected the external portion of the catheter was reduced to approximately 1 cm in length, its tip sealed with Plasticine and the hind quarters of the foetus returned to the uterus with the catheter in place. It was not found necessary to use an antispasmodic drug, and the uterine cavity was closed with silk thread and the overlying skin with suture clips. The doe was released and placed in a comfortable box with access to food and water. Three to five hours later the doe was again placed on the board, the wound re-opened and the foetus delivered. The doe was then bled from the heart and killed by injecting air into the heart. The occluded tip of the catheter in the foetal bladder was snipped off and any urine which had been formed was collected and weighed, and the results have been expressed as ml. without applying any correction for specific gravity. The slight error introduced in this way does not appear in clearance values, for these are computed from the total excretion of the solute measured.

New-born and young animals were either held in the hand with the catheter in place while the bladder was being allowed to fill, or were catheterized at 20–30 min intervals; adults were restrained on an animal board with an indwelling catheter. All studies were carried out with the animal in its natural state of hydration.

Urea and endogenous creatinine clearances were measured. The freezing-points of the urines and plasmas were determined before they were diluted for the analysis of urea and creatinine (Ramsay, 1949; Hervey, 1955), and the total osmolar concentrations calculated by dividing the depressions of the freezing-points by 0·00186. Urea in plasma and urine was determined by the method of Lee & Widdowson (1937), creatinine by a modification of the Jaffé reaction as described by Hawk, Oser & Summerson (1947), and chloride by the method of Sendroy (1937) or by that of Schales & Schales (1941).

RESULTS

A comparison of maternal and foetal plasma

Table 1 shows the average total osmolar, chloride, urea and creatinine concentrations in the plasma of maternal and foetal guinea-pigs at or very shortly before term. The differences were not significant, but differences in the same direction have been found in other species for osmolar and creatinine concentrations (McCance & Widdowson, 1953).

Comparison of maternal urine with the urine found in the foetal bladder

Table 2 contains comparisons of the total osmolar, chloride, urea and creatinine concentrations in maternal urine and in foetal urine found in the bladder at or shortly before term. Five other foetal urines have also been analysed without the corresponding maternal urines. The results have been given for each individual animal because the wide variation from one litter to another makes average figures of lesser value. There was considerably less variation between the individual foetuses within one litter. The osmolar concentrations were always much higher in the maternal than they were in the foetal urine, but the latter were generally hypertonic to serum, and much more concentrated than those of the human foetus or of the foetal lamb.

The concentration of chloride in both the maternal and foetal urines was very variable, but there was evidently a relation between the two urines, for they both tended to have a high or a low concentration together. In five instances when the maternal concentration was low, the foetus had a higher concentration of chloride in its urine than the mother.

The concentration of urea was always higher in the maternal urine, and this was largely responsible for the differences in total osmolar concentration. In nine out of the fifteen comparisons the concentration of creatinine was higher

TABLE 1. The total osmolar, chloride, urea and creatinine concentrations in the plasma of foetal guinea-pigs and their mothers

	No. of comparisons	Foetus		Mother	
		Average	S.D.	Average	S.D.
Total osmolar concentration (m-osmole/l.)	12	292	12·3	297	10·9
Chloride (m-equiv/l.)	8	99	7·1	102	4·1
Urea (mg/100 ml.)	13	30·4	6·97	33·7	8·08
Creatinine (mg/100 ml.)	16	1·6	0·52	1·45	0·48

TABLE 2. A comparison of the total osmolar, chloride, urea and creatinine concentrations in the urine found in the bladders of foetal guinea-pigs and of their mothers

Litter no.	State of* maturity of foetus	Total osmolar concentration (m-osmole/l.)		Chloride (m.-equiv/l.)		Urea (mg/100 ml.)		Creatinine (mg/100 ml.)		Urea: creatinine ratio	
		Foetus	Mother	Foetus	Mother	Foetus	Mother	Foetus	Mother	Foetus	Moth
1	M.	(*a*) 338	1354	149	240	187	587	114	92	1·64	6·38
		(*b*) 376		130		228		100		2·28	
		(*c*) 564		123		138		122		1·14	
2	M.	(*a*) 371	1306	158	310	244	1950	22	168	11·10	11·6
		(*b*) 402		188		208		38		5·47	
3	M.	(*a*) 328	912	64	25	35	1315	230	138	0·15	9·55
		(*b*) 339		28		46		276		0·16	
		(*c*) 360		39		38		236		0·16	
4	M.	387	1157	136	517	290	500	32	75	9·05	7·15
5	M.	246	946	61	42	66	688	—	—	—	—
6	M.	(*a*) 336	1114	44	138	195	380	81	98	2·41	3·85
		(*b*) 348		58		130		162		0·80	
		(*c*) 393		69		300		144		2·08	
7	M.	242	—	107	—	358	—	106	—	3·38	—
8	S.I.	304	1602	16	41	—	—	195	83	—	—
9	S.I.	—	—	—	—	100	1000	28	63	3·57	15·8
10	S.I.	—	—	134	113	220	760	38	65	5·80	11·7
11	S.I.	344	—	—	—	365	—	68	—	5·38	—
12	S.I.	320	—	80	—	226	—	77	—	2·94	—
13	S.I.	(*a*) 252	—	35	—	291	—	36	—	8·13	—
		(*b*) 302		—	—	131	—	39	—	3·38	—

*M., mature, approx. 70 days foetal age; S.I., slightly immature, approx. 60 days foetal age.

in the foetal than in the maternal urine. The most striking thing about these results for the urine found in the bladders of foetal guinea-pigs, however, was the variability of the concentration of urea and creatinine in them. Unlike the chloride, there seemed to be no relation whatever between the composition of the maternal and the foetal urine in this respect. In the urines from four of the fourteen full-term foetuses the concentration of creatinine was higher than that of urea; in the other ten it was lower, and the urea: creatinine ratios varied from 0·16 to 11·1. In the slightly immature animals the urea was always higher, as though this rising concentration of creatinine was something that happened at or about term. Samples of urine found in the bladders of full-term foetal guinea-pigs have been treated with Lloyd's reagent (Hare, 1950; Owen, Iggo, Scandrett & Stewart, 1954), and this removed all the chromogen which gave a colour within 20 min with the Jaffé reagents. In this respect, therefore, the chromogen behaved like 'true' creatinine.

Rates of urine formation and urea and creatinine clearances

Very often no measurable amounts of urine could be collected from the foetus during the 3–5 hr after catheterization. Measurements of urine flow were made in ten foetuses and these were of three degrees of maturity. Their volumes, separated into three age groups, are presented in Table 3. The foetal urine flow tended to decrease as gestation advanced, and at or about term it appeared to be about one-tenth the rate it had been four weeks earlier. The higher figure is of the order of magnitude obtained by Wells (1946) in the foetal rat at term (0·0025 ml./100 g body wt./min), but considerably less than the lowest rate reported by Alexander *et al.* (1955*b*) for the foetal sheep (0·004 ml./100 g body wt./min). A fall in the rate of urine flow during foetal life was evident in Alexander's studies.

In the first hour after birth the average urine flow was much higher than that of the foetus at term in spite of the fact that no fluid had been administered and that water was then being lost by the respiratory tract and skin. In the second and third hours after birth the flow of urine fell unless supported by the administration of oral fluids. This is not shown in the table. A further increase in urine flow took place during the first week of life and this was followed by a slower rise to adult levels. In the adult urine flow was often higher in the first half hour after catheterization than subsequently, and might then fall to a third or a quarter of its initial value.

From Table 3 it would also appear that the average concentration of urea in the plasma rose during the first week of life and then fell slowly towards the level characteristic of the normal adult (see also Table 1). A similar phenomenon has been observed in human infants, rats and pigs (McCance & Widdowson, 1947; Schultze, 1949; Halvorson & Schultze, 1950; McCance & Otley, 1951).

The urea and creatinine clearances were variable during foetal life and there was no definite trend in one direction or the other. The urea clearance increased more rapidly than the creatinine clearance during the first week of extra-uterine life with the result that the urea:creatinine clearance ratio rose to about 1·0 between the fourth and seventh days. After the first week the ratio fell to the level usually found during adult life. The creatinine clearance of the adults amounted to 0·25 ml./100 g/min, which is very close to the figure obtained by Dicker & Heller (1951) for the inulin clearance (0·23 ml./100 g/min).

TABLE 3. Development of renal function in the guinea-pig

		Plasma			Clearances		
Age	No. of animals	Urea (mg/ 100 ml.)	Creatinine (mg/ 100 ml.)	Urine flow (ml./100 g body wt./min)	Urea (ml./100 g body wt./min)	Creatinine (ml./100 g body wt./min)	Clearance ratios, urea : creatinine
Before birth							
40 days approx. gestation	1	29	1·72	0·002	0·003	0·015	0·20
	1	50	1·48	0·0016	0·002	0·006	0·33
60 days approx. gestation	1	45	—	0·00036	0·0004	—	—
	1	32	1·80	0·00047	0·0008	0·011	0·07
	1	35	1·25	0·00040	0·0019	0·010	0·19
	1	30	1·19	0·00229	0·0051	0·017	0·30
	1	44	1·38	0·00222	0·0124	0·069	0·18
70 days approx. gestation	1	20·5	1·38	0·0003	—	0·004	—
	1	40	1·40	0·0002	—	0·003	—
	1	32	1·20	0·00085	0·00265	0·018	0·15
After birth							
New-born	7	35	1·40	0·002	0·006	0·042	0·13
0–4 days	5	43	1·20	0·005	0·096	0·125	0·77
4–7 days	7	46	0·85	0·01	0·20	0·18	1·11
7–19 days	5	41	0·85	0·015	0·16	0·23	0·70
Adult	7	39	0·80	0·03	0·16	0·25	0·63

Urine 'found' and 'formed'

The urine 'found' in the full-term foetal bladder at the beginning of an experiment has already been described (see Table 2). It differed in appearance from the urine 'formed' *in utero* during the experimental periods. The urine 'found' at term had a dark amber colour, whereas that freshly 'formed' *in utero* was colourless or a very pale amber colour. Analysis (Table 4) revealed that this difference probably extended to chemical composition as well as to total osmolar concentration. Both physical and chemical differences were less obvious in younger foetuses. At 60 days the concentration of creatinine in urine 'found' in the bladder was somewhat greater than that 'formed' *in utero* during observation while at term the concentration was a great deal higher. The highest concentration of creatinine in a urine 'found' at term was 320 mg/100 ml. and the urine 'formed' immediately afterwards *in utero* contained only 19 mg/100 ml. The concentration of creatinine in the urine 'found' in the bladder increased with foetal maturity, but there seemed to be no correlation with age in 'formed' urine.

TABLE 4. Urea and creatinine concentrations and the total osmolar concentration of (*a*) urine found at the first catheterization in 40, 60 and 70 day foetuses, and (*b*) urine formed during the experiments by foetuses of the same ages

Urine 'found' in foetuses of	Urea (mg/100 ml.)	Creatinine (mg/100 ml.)	Urea : creatinine ratio	Total osmolar concentration (m-osmole/l.)
40 days approx.	88 (1)	17 (1)	5·2 (1)	—
60 days approx.	222 (6)	48 (6)	4·6 (6)	304 (5)
70 days approx.	216 (20)	140 (20)	1·5 (20)	368 (13)
Urine 'formed' during the experiments				
40 days	57 (2)	9·4 (2)	6·1 (2)	—
60 days	134 (4)	32 (4)	4·2 (4)	256 (2)
70 days	100 (1)	25 (1)	4·0 (1)	250 (1)

The number of observations is shown in parentheses.

DISCUSSION

The plasma

If the chromogen in foetal plasma is all creatinine, this substance may be one of those which does not equilibrate freely across the placental membranes. The present work suggests that if there is some other chromogen in the plasma it is not excreted in the urine. Further study is required.

The rise in the concentration of urea in the body fluids after birth, and its subsequent fall, would appear to be similar to the findings which have been established in other animals and in man. It is due to the rate of urea production, from tissue destruction or from the metabolism of ingested amino acids not used for growth, temporarily exceeding the amount of urea which the immature kidney excretes. With so many variables it is not surprising that there should be considerable differences between species and individuals in the extent to which the blood urea may rise. The matter has been investigated in some detail in pigs (McCance & Widdowson, 1956).

The urine

The urine found in the bladder of the foetal guinea-pig differed from the urine formed subsequently, and also from the urine which has been taken from the bladder of the foetal lamb or human being, in that it was usually hypertonic to the serum, and also in the very high concentrations of creatinine sometimes found in it and the correspondingly low urea: creatinine ratios. Indeed, these latter findings differentiate it from any other urines previously taken from the mammalian bladder or passed spontaneously by such an animal. They will be of value in future considerations of renal function *in utero* and they add interest now to the observations already made on the foetal rat (Wells, 1946). The composition of the urine formed immediately after birth, and of that formed after catheterization, suggests that the urine found in the bladder may have been secreted very slowly in the foetal guinea-pig, especially near term. If the rate of formation of urine were extremely slow near term and the tubules and collecting apparatus, including perhaps even the bladder, impermeable to creatinine but permeable, as at all ages to some extent, to urea, the findings could be explained; to postulate that creatinine was excreted into the urine seems less reasonable. If this suggestion is correct, there must also be considerable reabsorption of chlorides from these glomerular filtrates, but most very young animals have low chloride clearances and there is nothing inherently impossible in this part of the theory. The way in which the chlorides in the maternal and foetal urines varied in parallel with each other may be a point of some significance.

It is impossible to say at present whether the urine formed after catheterizing the foetuses or any of these guinea-pigs is a strictly normal product or not. The rate of formation may have been changed by the operative interference, although as a rule it is thought not, but further work on the young guinea-pig both before and after birth is in progress, and it is hoped to resolve some of the difficulties which have been discussed.

SUMMARY

1. The uterus of the pregnant guinea-pig was incised and female foetuses at three stages of maturity were catheterized and returned to the uterus for 3-5 hr. Urine flow, and urea and creatinine clearances were measured.

2. The rate of urine flow, expressed per unit of body weight, declined between 40 days of gestational age and term, and rose rapidly immediately after birth.

3. The urine found in the bladder of the foetus at or near full term was generally hypertonic, with an average total osmolar concentration of 368 m-osmole/l. The concentration of chloride in the foetal urine was very variable and tended to rise and fall with the concentration in the urine of the mother. The concentration of creatinine was sometimes extremely high, greater even than the concentration of urea.

4. These unusual urea:creatinine ratios were not found in the urine formed after catheterization, or in new-born, growing or adult animals.

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ANTIDIURESIS IN ADULT AND OLD RATS

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Friedman, Hinke & Friedman (1956) have shown that the injection of hypertonic sodium chloride solution into the common carotid arteries of non-anaesthetized hydrated rats produced a smaller antidiuretic effect in senescent than in younger animals. They attribute the decrease of the antidiuretic effect in old rats to failure of the neurohypophysis to react to the stimulation of hypertonic saline solutions.

The accidental finding, during a series of routine assays, that intravenous injections of known amounts of vasopressin into old rats under ethanol anaesthesia did not produce the expected antidiuretic effect, led us to reinvestigate the problem with a view to determining whether the decreased antidiuretic response reported by Friedman and his associates was really due to a decreased responsiveness of the neurohypophysis to osmotic stimuli or rather to a decreased sensitivity of the kidneys to the antidiuretic hormone.

METHODS

Old white rats of about 350 days and an average body weight of 370 g (range 325–415 g) were compared with control adult animals, about 100 days old with an average weight of 250 g (range 210–270 g). The old rats had been reared either on a diet containing 13·7 % of protein (diet D 41) or on one containing 20 % protein (diet D 86). Control rats had been fed on diet D 41 only. The role of the protein content of the diet on renal function in rats has been reported previously (Dicker, 1949).

For pressor assays, a modification of Dekanski's method (1952) was used. The blood pressure was recorded from the femoral artery. A carotid artery, as well as a vein, was cannulated, so that it was possible not only to assess the effect of an intracarotid hypertonic sodium chloride solution on the blood pressure, but also to estimate it in terms of intravenously injected vasopressin. A (2 + 2) dose assay of up to four groups was used. The results were calculated by standard statistical methods (B.P. 1953); the errors fell well within the limits stated previously (Dicker & Nunn, 1957).

Antidiuretic effects following intracarotid injections of various solutions in non-anaesthetized hydrated rats were estimated as follows: the common carotid artery and the bladder were cannulated under light ether anaesthesia; 1 hr after the operation, when the animal had entirely recovered from anaesthesia, 5 ml. water/100 g was given by stomach tube. When the water diuresis was well established, vasopressin (Pitressin, Parke, Davis and Co.) or hypertonic sodium chloride solution was injected into the carotid artery. The antidiuretic effect was estimated according to Friedman *et al.* (1956) as: $(U2 - U1)/U1 \times 100$, where $U1$ = rate of urine flow (ml./min) before injection and $U2$ = rate of urine flow after the injection. Antidiuretic assays were carried out on rats, under ethanol anaesthesia, kept with a constant water load (Dicker. 1953).

RESULTS

Validity of pressor assays in adult and old rats

Rats fed on a high protein diet, D86, were somewhat heavier than those of a similar age fed on diet D41. The body weights were 387 ± 10·20 g (13) and 370 ± 9·41 g (19) respectively. There was no statistical difference between the results of assays of standard amounts of pressor activity of vasopressin, performed either on old rats fed on high or moderately high protein diets, or on control animals (Table 1).

TABLE 1. Assay of pressor activity in adult and old rats

Type of rat	Diet	Stated potency of pressor activity (m-u./0·1 ml.)	Estimated amount of pressor activity (m-u./0·1 ml.)	Percentage recovery	Fiducial limits of error (%)
Old	D86	6·7	7·6	113·4	116–89
		12·0	10·1	84·2	127–78
		17·5	15·3	87·4	118–85
Old	D41	6·5	7·4	113·8	124–96
		25·0	24·6	98·4	110–91
		40·0	38·0	95·0	110–90
Control	D41	7·5	8·5	113·3	130–86
		10·0	9·2	92·0	115–80
		20·0	18·0	90·0	120–77

Validity of antidiuretic assays in adult and old rats

There was a marked difference between the results of assays of the antidiuretic activity of vasopressin according to whether old or normal adult rats were used. In six adult rats the estimated potency of known amounts of antidiuretic activity was 95% (range 85–115%). In old rats, however, especially those fed on a high protein diet (D86), there was a great variability in the renal responses following intravenous injections of known amounts of vasopressin. In six of these animals the mean estimated potency was 68% only (range 35–126%) of the stated potency. This showed clearly that old rats cannot be used reliably for the estimation of antidiuretic activity.

Release of pressor activity

There was no difference between the rise of blood pressure following the intravenous injections of graded doses of vasopressin in old rats fed on a high protein diet and in normal adult animals (Fig. 1*A*); furthermore, there was no difference in the pressor response to injections into the carotid artery of a 3% NaCl solution in old and adult rats (Fig. 1*B*). This would suggest that in both old and adult rats the blood vessels were equally sensitive to the injection of vasopressin, and that the neurohypophyses of old and of adult rats responded to the stimulation of hypertonic saline solutions to the same extent. It will be seen (Fig. 1*B*) that the intracarotid injection of 0·3 ml. of 3% sodium chloride produced in all animals, irrespective of their ages, a rise of blood pressure equivalent to that observed after an injection of 3·0 m-u. vasopressin (Fig. 1*A*). There was no appreciable difference between the reactions of old rats, whether fed on a high or on a moderately high protein diet. Intracarotid injections of hypertonic solutions of glucose (18 g/100 ml.) failed to affect the blood pressure.

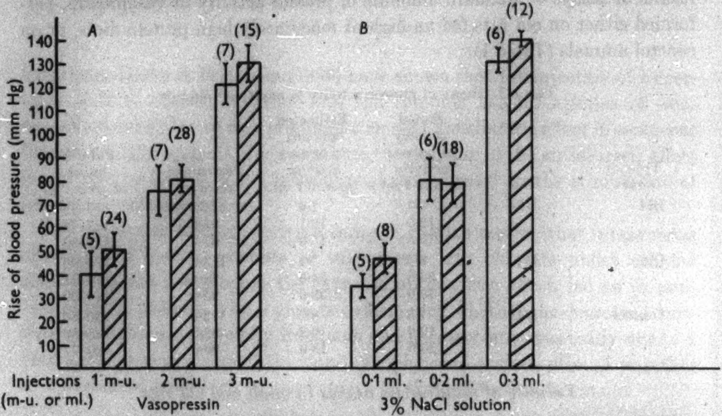

Fig. 1. Rise of blood pressure following intracarotid injections of (*A*) vasopressin or of (*B*) a 3% NaCl solution in control (open columns) and in old rats (hatched columns). Vertical lines = S.E. of means; in brackets, number of experiments.

Renal responses to intracarotid osmotic stimulation

Intracarotid injections of vasopressin produced the same degree of antidiuresis as intravenous injections; hypertonic saline solutions, however, had an antidiuretic effect only if injected into the carotid artery. Injections of 0·1 ml. of an 18% (w/v) glucose solution into either the carotid artery or a vein had no effect on the urine flow.

About the same antidiuretic action was found after injection of 0·3 ml. of a 3% NaCl solution and of 3·0 m-u. vasopressin in adult and in old rats (Table 2); it will be seen, however, that the actual antidiuretic effect following the injection of 3·0 m-u. vasopressin or of a 3% NaCl solution, was markedly smaller in old than in adult rats (Table 2). This suggested that the kidneys of old rats responded less well to the antidiuretic stimulation of endogenous or exogenous vasopressin than those of younger animals, and provided an explanation for the previous findings that old rats, and especially those fed on a high protein diet, were not suitable for the accurate assay of antidiuretic activity.

Urine concentration in old rats

Old rats fed on a moderately high protein diet (D41) and adult rats were deprived of water for 24 hr. Urine samples were collected during the last 14 hr of dehydration for freezing-point estimations. Old rats were not able to concentrate their urine to the same degree as control animals (Table 3).

TABLE 2. Comparison of antidiuretic reactions in old and adult rats

$$\text{Antidiuretic effect} = \frac{U2 - U1}{U1} \times 100$$

Adult rats		Old rats	
After 3·0 m-u. vasopressin	After 3% NaCl soln.	After 3·0 m-u. vasopressin	After 3% NaCl soln.
$85{\cdot}9 \pm 3{\cdot}6$*	$79{\cdot}9 \pm 4{\cdot}9$	$52{\cdot}8 \pm 7{\cdot}6$	$39{\cdot}4 \pm 10{\cdot}80$
(4)	(7)	(8)	(7)
$t = 0{\cdot}802$		$t = 0{\cdot}957$	
$0{\cdot}4 > P < 0{\cdot}5$		$0{\cdot}3 > P < 0{\cdot}4$	

All injections were given into the common carotid artery.

* Means ±S.E.; number of experiments in brackets.

TABLE 3. Urine concentration in old and adult rats

	Old rats	Adult rats
Mean body weight (g)	385	255
Mean urine excretion* (ml./100 g/24 hr)	0·97	0·62
Δ (range)	$-3{\cdot}90$ to $-4{\cdot}18$	$-4{\cdot}80$ to $-5{\cdot}12$

* Urine excretion was measured during the last 14 hr of water deprivation; number of rats in each group, 6.

DISCUSSION

Ginsburg & Brown (1957) observed an increase in urine flow without 'any indication of an antidiuretic effect' following injection of hypertonic saline into the internal carotid arteries of rats. The present investigation, however, agrees with the findings of Friedman *et al.* (1956) that an injection of hypertonic sodium chloride solution into the common carotid artery has an antidiuretic effect in the rat, as in the dog (Verney, 1947), but differs from those of Friedman *et al.* in one important respect. Friedman and his associates attributed the smaller antidiuretic effect observed in old rats after an intracarotid injection of a hypertonic saline solution to 'a decline in neurohypophyseal function with age', though they did not measure the actual excretion of the hypophyseal hormone. The present observations show, however, that in both old and adult rats an intracarotid injection of hypertonic saline solution produces the same release of pressor activity, and consequently the response of the neurohypophysis to osmotic stimulation does not appear to diminish with age.

The smaller antidiuretic effect following intracarotid injection of hypertonic solutions in old rats is therefore presumably of renal origin. It will be remembered that in man there is a progressive decrease in the inulin and diodone clearances (Shock, 1946) accompanied by a decrease of the concentrating ability of the kidneys (Lewis & Alving, 1938). From the present series of experiments it would appear that the antidiuretic effect of a fixed dose of vasopressin was smaller in older than in younger rats. This agrees with the facts that the assay of known amounts of antidiuretic activity proved unreliable in old rats and that there was a decrease in the ability of the kidney to concentrate the urine during water deprivation.

SUMMARY

1. Injections of hypertonic NaCl solutions into the common carotid artery in rats produce, as in dogs, a release of the neurohypophyseal hormone, which results in both a pressor and an antidiuretic response.

2. The pressor response following the intracarotid injection of hypertonic NaCl solution is the same in old and in adult rats.

3. In contrast with adult rats, in old animals there is a great variability of the antidiuretic response following the intravenous (or intra-arterial) injection of vasopressin. This makes assays of antidiuretic activity in old animals inaccurate and unreliable. Old rats do not concentrate their urine to the same degree as control animals.

4. There is no evidence of a decline with age in neurohypophyseal response to hypertonic stimulation; the smaller antidiuretic effect observed after intracarotid injection of hypertonic NaCl solution in old rats appears to be of renal origin.

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TEMPORAL AND SPATIAL SUMMATION IN HUMAN VISION AT DIFFERENT BACKGROUND INTENSITIES

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The present experiments were undertaken as part of an investigation of the suggestion made by Rose (1942, 1948) and de Vries (1943) that human visual performance is limited by the inevitable fluctuations in the numbers of quanta absorbed in the retina. In a previous paper (Barlow, 1957) it was shown that this idea (modified by assuming that there is also a weak intrinsic source of noise) leads to theoretical curves which fit experimental determinations of increment threshold made with a short duration small area test stimulus superimposed upon a large uniform adapting field. It was also shown that big changes in the amount of temporal and spatial summation occur when the background intensity is changed, with the result that when thresholds are determined with a long duration large area test stimulus the experimental points deviate from the appropriate theoretical curve and tend to obey the Weber law instead.

In following up this finding there were two objectives. The quantum fluctuation hypothesis predicts that the increment threshold intensity should be inversely proportional to the square root of the area and duration of the stimulus, and the first object was to find whether the occurrence of these laws fitted in with the hypothesis: the results show that the predicted laws of summation do hold over certain ranges, but when they hold the actual values of the thresholds are higher than the theory predicts. The second objective was to determine the parameters α and τ which were introduced in the previous paper; these are the area and time over which quanta absorbed from the background light are liable to be confused with those absorbed from a short duration small area stimulus light, and here the results obtained are disappointing, for one can only derive lower limits to these quantities. On the other hand, the results do show up the complicated interrelations between temporal and spatial summation and background intensity; tentative explanations of these effects, and of the failure to perform up to the quantal fluctuation limit, are put forward.

METHODS

The apparatus and its calibration have been described in the previous paper. It provided a background or adapting field of 13° diameter which was centred 6° 30′ from the fovea in the inferior nasal quadrant of the visual field of the subject's right eye. The stimulus, or test flash, was added to the centre of the background field, and consisted of a disk subtending from 6′ to 5·9° at the subject's eye. Its duration varied from 7 to 1000 msec and was measured with photocell and oscilloscope. The intensity of the background was controlled by neutral filters, and that of the stimulus by neutral filters and a wedge which was adjusted by the subject. In all the experiments reported here an Ilford 603 (blue-green) filter was placed in both the background and stimulus pathways. The light passing this filter had a peak energy at 497 mμ and quantities of light are expressed as the number of quanta of wave-length 507 mμ (quanta/sec. degrees^2 for intensities) which would have had the same scotopic luminosity as the light in question. The light entered the eye through an artificial pupil 2 mm in diameter.

The same two subjects were used as in the previous experiments, and the same technique for determining threshold; the subject adjusted the wedge, with the stimulus repeating automatically at about 1 per 3 sec, until he considered that it was usually just visible. This seemed to correspond approximately to the '80% seen' intensity of frequency of seeing curves. The usual procedure was to determine a threshold at each area or duration of stimulus, then change to the next area or duration in the series; having completed the series in one order, it would then be repeated in reverse order. Each experimental run was preceded by a period of dark adaptation, and whenever the background intensity was changed the subject was allowed a few minutes to adapt to it.

RESULTS

Fig. 1 shows the over-all change in combined spatial and temporal summation for a range of background intensities from zero to 10^9 quanta/sec. degrees^2, using the fixation point so that the test stimuli were centred 6° 30′ from the fovea. The measure of summation that was used was the ratio of the threshold *quantity* of light for a short duration small area stimulus to the threshold *intensity* using the longest duration and largest area available; this quantity represents a solid angle multiplied by a duration, and its logarithm is plotted as ordinate. The results for both subjects are shown, and each point is the mean of the log. of up to four estimates of the ratio; the pair of thresholds required for a single estimate were determined within a few minutes of each other, but the different estimates were made over a period of several months. It will be seen that the amount of summation decreases continuously as the background intensity is raised over the whole of this range. At low intensities B.N. shows rather less summation than H.B., but this difference disappears at high intensities. The straight line drawn in the figure has a slope of $-0{\cdot}25$, and therefore represents the law

$$\Delta I/\Delta N \propto I^{\frac{1}{4}},$$

where ΔI is threshold intensity, ΔN threshold quantity, and I the background intensity. This is an empirical finding with no theoretical basis, and it may depend to some extent on the stimulus conditions used.

One other feature of Fig. 1 is worth comment. Stiles's evidence (1939, and personal communication) suggests that cones (the π_4 mechanism) are probably the receptors subserving threshold at field intensities above 10^6 or 10^7 quanta/sec. degrees^2 under the conditions of these experiments, and this conclusion is consistent with some observations of the relative effectiveness of background lights of different colours in raising the threshold of the superimposed stimulus. Below this intensity rods must be operating, so that the first five pairs of points, up to and including the pair at log $I = 5{\cdot}7$, depend upon the rod mechanism, whereas the remaining three probably depend on the cones. There is no break in the curve at this point (though there might be with different colours for stimulus and background lights), but the present result is sufficient to prove that the decrease in summation with increase in background intensity applies to the cone mechanisms as well as to the rod mechanisms.

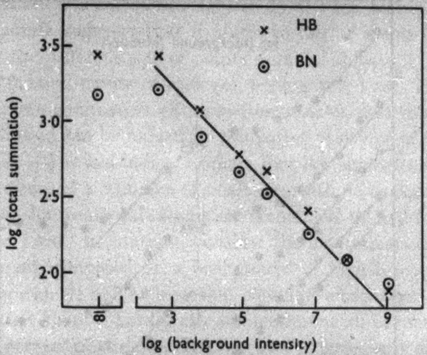

Fig. 1. The effect of background intensity on the amount of combined spatial and temporal summation up to 1 sec and 19 degrees^2. The measure of total summation whose logarithm is plotted as ordinate is the ratio of threshold *quantity* of light (for 7·6 msec, 0·0077 degrees^2. stimulus) to threshold *intensity* (for 935 msec, 19 degrees^2 stimulus).

If the product of solid angle and time, whose logarithm is plotted in Fig. 1, is to be free of arbitrary dependence upon particular conditions, the threshold *quantity* of light must be measured with a stimulus of area and duration within the ranges where complete temporal and spatial summation occur (Bunsen-Roscoe and Ricco ranges), and the threshold *intensity* with a stimulus of area and duration large enough for summation to have ceased. If these conditions are not met, the ratio of thresholds will depend upon the actual area and durations of stimuli used. For Fig. 1 the threshold quantities were estimated using a flash of 0·0077 degrees^2 (6′ diameter) and 7·6 msec duration; these cannot be assumed to lie within the ranges of complete summation, especially at high background brightnesses. Likewise it is uncertain that all summation has ceased for the stimulus of 19 degrees^2 (4·9° diameter) and 1 sec duration, used for measuring threshold intensity. For this reason thresholds were determined at intermediate durations and areas at the various background intensities on one subject. It was hoped that these results would also show whether the changes in total summation involved changes in both temporal and spatial summation, and whether the changes took place in the upper limits of complete summation (Bunsen-Roscoe and Ricco ranges), or whether the amounts, or ranges, of partial summation were involved.

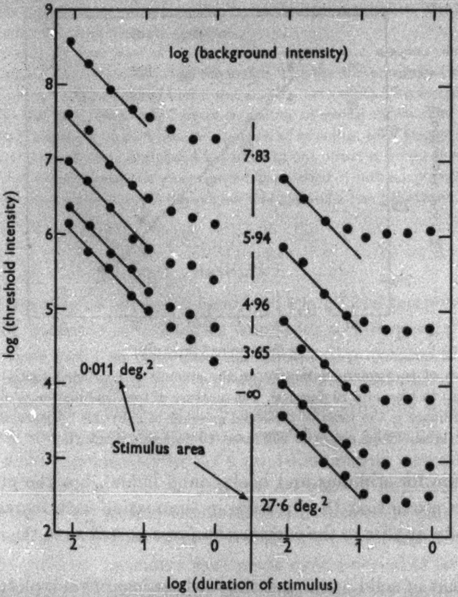

Fig. 2. Temporal summation. Log (increment threshold intensity) plotted against log (duration) for a small (0·011 degrees^2) and a large (27·6 degrees^2) area of stimulus at five different background intensities. The straight lines have slope −1 and are continued up to 0·1 sec. Intensities in quanta [507 mμ]/sec. degrees^2; durations in sec.

Fig. 2 shows an experiment in which the increment threshold was measured for eight durations of test stimulus at five different background intensities (zero to $6{\cdot}8\times10^7$ quanta/sec. degrees^2 entering the eye), using test spots having a small diameter (7·1′; left half of figure) or a large diameter (5·9°; right half of the figure). Each point is the average of two settings, the repeat series being done in reverse order. The points at one background intensity were all done on the same day, and they belong to the same subject; the sets of points at different backgrounds were done at different sessions, but within a week of each other.

Consider first the set of determinations made with zero background and a small test spot (the bottom set of the left half of the figure). The duration was increased from 8·5 to 930 msec and this caused a drop in threshold intensity from $1{\cdot}35 \times 10^6$ to $2{\cdot}1 \times 10^4$ quanta/sec.degrees^2; an increase of duration by a factor of 110 caused a decrease of threshold by a factor of 64, and the actual number of quanta required increased less than twofold from 130 to 220. Temporal summation is not quite complete up to 1 sec, and inspection of the intermediate points suggests that it starts to fail at about 0·1 sec. To aid comparison of the different sets of points a line of slope -1 has been drawn through the left-hand points of each set, ending at 0·1 sec. This line corresponds to complete summation (Bunsen-Roscoe law), and the actual law of summation followed can be judged by comparing the slope of the points with that of the line; $-\frac{1}{2}$ would indicate square root law summation; 0, zero summation. In the case of a stimulus of small area with no background illumination one can say that temporal summation is complete up to 0·1 sec, and nearly complete up to 1 sec. In contrast, consider the determinations made with a large stimulus superimposed on a background of high intensity (top set of right half of figure). It will be seen that complete summation stops at about 30 msec, and that there is hardly any summation at all beyond this point, so that here the increase of stimulus duration by 110 times only caused a 5 times decrease of threshold intensity, and the actual number of quanta required increased from $1{\cdot}4 \times 10^6$ to $3{\cdot}2 \times 10^7$ quanta, an increase by more than 20 times.

Fig. 3 shows log. increment threshold plotted against log. area of stimulus at five different background intensities and two durations of stimulus. The determinations at each background intensity were done in the same session as those at the same intensity in Fig. 2. The line of slope -1 indicates complete spatial summation (Ricco's Law), and it has been drawn up to 0·4 degrees^2. The actual amount of spatial summation can be judged from the slope of the sets of points. The display of spatial summation in Fig. 3 is similar to the display of temporal summation in Fig. 2, and it will be seen that the results are also analogous.

The general features of the complicated interrelations which these figures reveal are as follows. Temporal summation varies from complete (Bunsen-Roscoe Law valid) to zero (threshold intensity independent of duration) and the factors which reduce it (i.e. lessen the downward slope of the points) are (i) increase of stimulus duration, (ii) increase of stimulus area, and (iii) increase of background intensity. Exactly the same factors decrease the amount of spatial summation, though this never quite comes down to zero, and in consequence the lowest threshold intensities here recorded are higher than the values found with larger stimuli (e.g. Denton & Pirenne, 1954). Looking at the points where the degree of summation changes one sees that the upper limit of complete temporal summation is about 0·1 sec, and the range is probably slightly curtailed by increasing the background intensity: this effect is more pronounced with large area stimuli, when the upper limit comes down to about 0·03 sec. The upper limit of complete spatial summation (Ricco's Law valid) is about 0·4 degrees^2 with short durations at low backgrounds, but it is reduced to about 0·1 degrees^2 by raising the background intensity: increasing the duration of the stimulus reduces it still further to a value of about 0·025 degrees^2. Beyond the ranges of complete temporal and spatial summation there are ranges of partial summation which, within a variable range, approximate to the square-root laws predicted by the quantum fluctuation hypothesis. The upper limits of partial summation have not been reached under all conditions, but it can be seen that these ranges too are curtailed by the factors which reduce the ranges of complete summation—increase of stimulus area and duration, and background intensity.

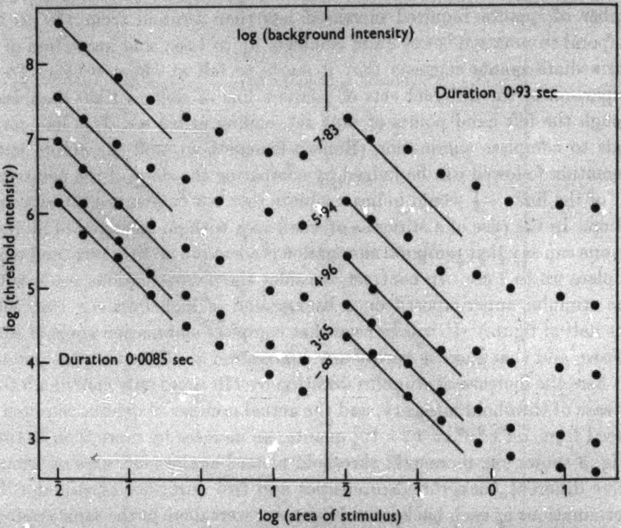

Fig. 3. Spatial summation. Log (increment threshold intensity) plotted against log (area) for a short (8·5 msec) and long (930 msec) duration of stimulus at five different background intensities. The straight lines have slope −1 and are continued to 0·4 degrees^2. Intensities in quanta (507 mμ)/sec.degrees^2; areas in degrees^2.

The two sets of curves of Fig. 2 were obtained using the largest and the smallest values for the area of the test stimulus. It would obviously be possible to obtain similar curves for intermediate sizes of the stimulus, and similarly one could use intermediate stimulus durations to get curves of spatial summation like those of Fig. 3. Complete sets of thresholds at all combinations of stimulus area and duration have been determined at two background intensities, and these show that curves of temporal and spatial summation at intermediate values of area and duration lie between those taken at the extreme values which are shown in Figs. 2 and 3. No special features appear to be associated with particular durations or areas of test stimulus.

These complicated phenomena become more coherent if it is pointed out that the three factors which hinder further summation—increase of area or duration of the stimulus, or increase of the background intensity—all decrease the ratio $\Delta I/I$ at threshold. Hence as $\Delta I/I$ is reduced it becomes less susceptible to further lowering by increase of the area of duration of the stimulus.

DISCUSSION

Previous results

Figs. 2 and 3 show that temporal and spatial summation in human vision are complex, interrelated phenomena which are not adequately described by the simple empirical laws (Bunsen-Roscoe, Ricco, and Piper) except within certain rather narrow ranges. The shortcomings of these laws are fairly widely known, and the conditions under which they are valid are discussed by Graham & Margaria (1935) and Graham, Brown & Mote (1939). The interrelations between temporal summation and spatial summation do not, however, appear to have been sufficiently appreciated, though they have been reported in the literature. Piéron (1920) stated that there were reciprocal relations between the duration of the stimulus and the amount of spatial summation, and between the area of the stimulus and the amount of temporal summation. Graham & Margaria (1935) studied this effect and showed that the area of the test stimulus had a big effect on temporal summation. Their curves show clearly that the limit of complete temporal summation (Bunsen-Roscoe Law) is reached earlier with a large than with a small stimulus area, and that the reduction of threshold intensity brought about by increasing the duration of stimulus from under 1 msec to over 0·5 sec is greater when stimulus area is small than when it is large. Baumgardt (1947) obtained similar results, and Graham and Margaria's main conclusions are confirmed by the present findings, but there are some discrepancies. They found deviations from the Bunsen-Roscoe Law at durations as short as 2 msec, whereas deviations do not appear in the present results until just below 0·1 sec. Furthermore, they found a large range of partial summation for the large stimulus, and only a small range for the small stimulus, whereas the reverse is true in the present results. The reason for these discrepancies is not clear, but they might result, in part at least, from the fact that they were using a retinal region 15° from the fovea, as against 7° in this paper. If this is the correct explanation, the retina closer to the fovea must be considerably more efficient at temporal summation than the more distant periphery.

The fact that an increase in the intensity of a background reduces the degree of spatial and temporal summation for a test flash superimposed upon it was shown by Stiles & Crawford (1934). Graham & Kemp (1938) showed the effect on temporal summation in rather greater detail, and Blackwell (1946) showed the effect on spatial summation in a very thorough study, but the interpretation of his findings is complicated by the use of a free-roaming eye so that the image of the test area could fall on any retinal region. Willmer (1954) showed that the apparent brightness of a small, foveally viewed, test patch was increased more by increasing its area at low intensities than at high intensities, which appears to be due to a change in the amount of spatial summation. The observations of Van der Brink & Bouman (1954) and Gregory (1955) also indicate changes of summation with intensity.

The effect of background luminance on summation must be closely related to the changes in summation during dark-adaptation which have been found by Craik & Vernon (1941), Arden & Weale (1954), and Rushton & Cohen (1954), though Wald (1938) failed to observe any such effect. Another observation which may be related is that of Beitel (1934, 1936), who observed the effect of one spot upon the threshold for another placed near it, and found the threshold lowered (summation) if it was at a low intensity, and raised (inhibition) if it was brighter. This was probably early evidence of that complexity of retinal organization, foreseen by Baumgardt & Segal (1947) and since (Kuffler, 1953; Barlow, 1953) shown clearly in electrophysiological preparations, which makes the interpretation of psychophysical experiments in terms of summation alone inadequate.

In the present paper a retinal locus 6° 30′ from the fovea was used. This is not ideal for the study of spatial summation because the sensitivity of the retina is not uniform here. Pirenne & Marriott (1954) showed that the threshold for a small stimulus drops by 0·5 log units on moving from 3° 30′ to 9° 30′ from the fovea, the limits reached by the largest test spot used here. Pirenne & Marriott (1954) also compared the threshold for the two halves of a circular test field 11·3° in diameter centred 7° from the fovea, and found the threshold for the peripheral half only 0·21 log. units lower; for a 6° spot the difference would presumably be less, and the degree of non-homogeneity this indicates is probably not enough to obscure the results of the present paper. The main contribution of these results is to show up the interrelations between temporal summation, spatial summation, and background intensity, in a single retinal region.

Area and time

α and τ were defined (Barlow, 1957) as the area and time over which quanta absorbed from the background were liable to be confused with quanta absorbed from a short duration small area stimulus, and the present observations were made in the hope of obtaining values for them. It was thought at first that the product of area and time which is plotted in Fig. 1 could be identified with the product of α and τ. The argument for doing so is that threshold depends on the *quantity* of light exceeding a certain value, and that stimulus and background lights must be treated alike by the unit which ultimately responds to this quantity. Fig. 1 may be thought of as showing the factor by which the *intensity* of a long duration large area stimulus must be multiplied to obtain this *quantity*, and it should therefore be equal to $\alpha\tau$, the factor by which the background *intensity* must be multiplied to obtain the *quantity* of light, which is liable to be confused with the stimulus. This argument would be acceptable in the case of many simple physical systems, but in the case of the eye it is seen to be fallacious as soon as one considers the complexity of organization of the retinal units. The surround of a receptive field tends to inhibit the central zone so that when light is delivered to both synchronously it is less effective than when it falls on the centre alone (Kuffler, 1953; Barlow, 1953): now it is clear that light from the stimulus falling in this surround will *not* sum with light falling in the central zone, but it is possible that *fluctuations* in the number of quanta absorbed from the background *will* contribute to the noise from which the stimulus is to be distinguished.

It does not seem possible to get definite values of α and τ from these results and the best that can be done is to take the upper limits to the Ricco and Bunsen–Roscoe ranges as lower limits to their values. One can extend a stimulus up to these limits without raising the threshold number of quanta required, and when it has these values the number of quanta from the background contributing to the noise cannot be less than the number absorbed in that area and time. There are, however, many possible reasons for the true values of α and τ lying beyond the ranges of complete spatial and temporal summation, so that one cannot decide their true values from these experiments, nor can one tell whether they change with background intensity.

Quantum fluctuation hypothesis

In counting the quanta absorbed from the stimulus a certain number of quanta coming from the background will inevitably be counted with them, and according to the hypothesis to be tested the threshold corresponds to the limit set by the fluctuations in this number. The limit will be lower if this number is reduced, but at the same time the maximum possible number of quanta from the stimulus must be collected. Using stimuli which are sharply delimited in space and time (as in the present experiments) the best performance will clearly be obtained by 'sampling' the numbers of quanta absorbed over areas and durations matched to the area (A) and duration (T) of the stimulus. When A and T are increased, the 'sampling' area and time must also be increased, and the average number of quanta counted from the background will then be increased in direct proportion to the area and duration of the stimulus. The actual numbers absorbed on particular occasions will be distributed according to the Poisson distribution, to which an approximation is given by a normal distribution of standard deviation equal to the square root of the average number of quanta absorbed. Hence, if the threshold response is of constant reliability, $\Delta N \propto A^{\frac{1}{2}}T^{\frac{1}{2}}I^{\frac{1}{2}}$, where ΔN is the incremental quantity of light; or $\Delta I \propto A^{-\frac{1}{2}}T^{-\frac{1}{2}}I^{\frac{1}{2}}$, where ΔI is incremental intensity. The dependence on intensity is actually described better if $I+X$ is substituted for I (Barlow, 1957), where X is an intrinsic source of noise represented as 'dark light'. The expected law then becomes

$$\Delta I \propto A^{-\frac{1}{2}}T^{-\frac{1}{2}}(I+X)^{\frac{1}{2}} \tag{1}$$

The results shown in Figs. 2 and 3 do not fit this equation. It is true that increment threshold is in fact roughly proportional to $A^{-\frac{1}{2}}$ over a moderate range which varies in position with background intensity and stimulus duration. This is the range for which Piper's law holds, and it would be represented on this double logarithmic plot by a line of slope $-0{\cdot}5$. Similarly, there is a rather short range in Fig. 2, where increment threshold is proportional to $T^{-\frac{1}{2}}$. The validity of such square-root laws might be taken as some evidence for the quantum fluctuation hypothesis, but there is a serious objection. The law $\Delta I \propto (I+X)^{\frac{1}{2}}$ holds for small area short duration stimuli, and it fits (approximately) the left-hand points of Figs. 2 or 3. It will obviously only continue to hold for as long as these curves continue downwards parallel to each other; but, as has been pointed out, the laws of complete temporal and spatial summation break down at smaller areas and times when the background intensity is raised. It follows that the law $\Delta I \propto (I+X)^{\frac{1}{2}}$ does not hold beyond the ranges of complete summation, and it is only here that the $A^{-\frac{1}{2}}$ and $T^{-\frac{1}{2}}$ laws begin to hold. Equation 1 does not, therefore, hold for all three variables over any range of A, T and I.

In summary, then, square-root laws are found as predicted by the quantum fluctuation hypothesis, but the range for areas is variable, and the range for times short; furthermore, for values of A and T where $\Delta I \propto A^{-\frac{1}{2}}$ and $T^{-\frac{1}{2}}$, $\Delta I \propto (I+X)^{\frac{1}{2}}$ is untrue, and conversely for values of A and I where $\Delta I \propto (I+X)^{\frac{1}{2}}$ holds, $\Delta I \propto A^{-\frac{1}{2}}$ and $T^{-\frac{1}{2}}$ do not.

Since equation 1 is wrong, one or both parts of the hypothesis from which it is derived must be wrong: either a subject's responses are not of equal reliability under different conditions, or they are not limited solely by quantal fluctuations, or neither is true. The first possibility appears unlikely to be sufficient to account for the major deviations because (*a*) false positive responses do not occur noticeably less often where the deviations are greatest (*b*) deliberate attempts to affect the reliability of response have rather a small effect on the absolute threshold (Barlow, 1956). Furthermore, the slope of frequency of seeing curves is related to reliability of response, but this does not seem to vary greatly within the range of conditions covered here (see Blackwell 1946), though it does under some conditions (see Denton & Pirenne, 1954; Crawford & Pirenne, 1954). Other factors must, therefore, reduce efficiency of vision below the quantal fluctuation limit, and the present results show the conditions where the efficiency is greatest, and what factors decrease it. In the retinal region 6° 30′ from the fovea efficiency is highest for a stimulus about 40′–45′ in diameter (0·4 degrees^2) and about 0·1 sec duration, superimposed on a background of low intensity. Efficiency falls off when the added stimulus is changed in area or duration to values lying outside the Piper and $T^{\frac{1}{2}}$ ranges, and when the background intensity is raised. The following tentative explanations for these losses of efficiency are suggested.

Mismatching loss

Work on single retinal units in the frog (Hartline, 1940; Barlow, 1953) has indicated that the response depends on the total quantity of light falling in the central zone of the receptive field, and it is natural to suppose that fluctuations in the background light in an area at least as large as this will interfere with the detection of incremental stimuli. Hence when a stimulus is smaller than this area an unnecessarily large number of quanta from the background must be summed with it, thus raising the threshold above the optimum that would be attainable with a receptive field matching the stimulus. For a fixed size of receptive field efficiency will be highest for a test spot which exactly fills it, and will fall off when the spot is reduced in size. In a similar way, efficiency might be expected to drop off when the duration of a stimulus is decreased below the summation time (upper limit of Bunsen-Roscoe range). Whether there is also a loss when the stimulus is larger or longer than the summation time will depend upon other factors, such as the extent of the 'transmission' or 'screening' loss (see below).

Lateral inhibition loss

In the cat (Kuffler, 1953) and frog (Barlow, 1953) it has been shown that the annular surround of a receptive field acts in opposition to the central zone. This can lead to the paradoxical situation where a large stimulus spot evokes a weaker discharge than a smaller one. More recently (Barlow, FitzHugh & Kuffler, 1957) it has been shown that this lateral inhibition occurs when the retina is light-adapted and the stimuli are superimposed on a steady background, but does not occur when the background is turned off and the retina dark-adapted. Furthermore, even in light-adaptation, the inhibitory effect is less pronounced when brief stimuli are used. Thus the conditions (raising background intensity and increasing stimulus duration) which diminish spatial summation in human experiments are the conditions which accentuate lateral inhibition in animal experiments. The gap between the quantum fluctuation limit and the actual performance with long large stimuli might thus be attributed to lateral inhibition.

Transmission or screening loss

The two preceding causes of loss of efficiency are based upon known physiological mechanisms, and may suffice to explain losses when the stimulus is too small and short, or too long and large. They do not explain why there is a progressive loss of efficiency, with all except short small stimuli, when the background intensity is increased. Gregory's (1956) suggestion that additional 'neural' noise is added to the system between the receptors and the visual centres might provide an explanation, but only if noise is added *after* the point at which sensory adaptation occurs: if the noise was added *before* adaptation, it would cause greatest loss of information with the weakest signals, that is at low intensities. An alternative proposal is derived from that put forward by Baumgardt (1953) to reconcile the slope of frequency of seeing curves at absolute threshold with his two-quanta explanation of spatial and temporal summation. He suggested that the absorption of two quanta within a certain area and time caused an impulse in the optic nerve, but that two or more such impulses were required to cause a sensation. The new feature of this idea is that the threshold decision is not made in a single step, but in stages; this feature fits easily into a signal noise theory of threshold. The original idea leading to equation 1 required that the absorption of every single quantum should be signalled to some central point where the threshold decision was taken. According to the new idea the retina would 'screen' messages from the receptors, and only signal centrally when something occurred with a moderately low prior probability if there was no stimulus. If one such signal corresponded to a very low prior probability, one would be sufficient to allow a 'seen' response to be given without danger of excessive false positives, and in this situation 'probability summation' alone could occur (see Pirenne, 1956). If, on the other hand, each signal corresponded to an event of high prior probability, the conditions would return closer to those assumed for equation 1. In this way the idea that impulses in the optic nerve occur moderately often without any incremental stimulus being applied, links the 'probability summation' hypothesis with explanations of temporal and spatial summation as signal/noise discriminations of constant reliability.

A screening mechanism of this type might explain a number of puzzling aspects of visual function; for example, the failure of temporal and spatial summation when the intensity of the threshold increment becomes low relative to the background intensity (see p. 343), and the occurrence of Weber's law with long large stimuli (Barlow, 1957). Furthermore as well as explaining why efficiency drops when the background is increased, it may also show how the eye avoids overloading and maintains reasonable efficiency over such a very wide range of intensities. It should be noticed that explanations of loss of efficiency through screening are complementary to explanations in terms of specific physiological mechanisms, and do not conflict with them. For instance, lateral inhibition may be the means by which the screening level is raised when the background intensity is increased. At present, however, one can only suggest that Baumgardt's screening concepts might be a fruitful modification of the quantum fluctuation hypothesis.

In conclusion, then, the eye does not perform equally efficiently in the detection of test objects of different areas and durations superimposed on backgrounds of different intensities, and these variations of efficiency provide clues as to the physiological mechanisms of vision.

SUMMARY

1. Increment thresholds have been measured in a retinal region 6° 30′ from the fovea with test spots of varying sizes and durations superimposed on backgrounds of various intensities.

2. The total amount of summation (as measured by the ratio of lowest threshold quantity of light to lowest threshold intensity) decreases continuously as background intensity is raised: this decrease occurs in the photopic as well as the scotopic range.

3. The upper limit of complete temporal summation is decreased by increasing the area of the stimulus, and by increasing the background intensity.

4. The upper limit of complete spatial summation is decreased by increasing the duration of the stimulus, and by increasing the background intensity.

5. The increment threshold lies above the limit set by quantal fluctuations of the background light, except possibly for a single set of conditions: factors which might prevent attainment of the limit are suggested.

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ANION INTERACTION IN FROG MUSCLE

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When other anions are substituted for the Cl^- of a saline mixture bathing a muscle the irritability is increased (Chao, 1934). The twitch following stimulation is prolonged (Kahn & Sandow, 1955) and the 'active' state is more persistent (Hill & Macpherson, 1954). Although there is evidence that the membrane resistance is increased in the presence of foreign anions (Padsha, 1957), Edwards, Harris & Nishie (1957) failed to find an effect of the anions on cation exchange. Recently R. H. Adrian (unpublished) has reported at a meeting of the Physiological Society (March, 1957) that Cl^- movement from muscle is slowed by the foreign anions. The present results confirm that Cl^- movement is made slower by the anions NO_3^-, I^-, CNS^- and ClO_4^-. The same proves to be true of Br^- output. Comparisons have been made of Cl^- output in solutions respectively of Na or K salts of various anions.

METHODS

Sartorii from *Rana temporaria* or *R. esculenta* were treated with a solution containing a proportion of either ^{36}Cl or ^{82}Br. The Br samples were kept for at least 3 days before use so that the shorter-lived Br isotope should have decayed. The ^{36}Cl was obtained either as $H^{36}Cl$ or as $K^{36}Cl$, while the ^{82}Br was obtained as $NH_4{}^{82}Br$ and converted to K or Na salt by use of an ion-exchange column.

The solutions used for loading the muscle were either:

(*a*) a saline mixture with NaCl (or NaBr) 90, $NaHCO_3$ 30, KCl 3, $CaCl_2$ 2 mM; or

(*b*) KCl (or KBr) 120, $NaHCO_3$ 3, $CaCl_2$ 2 mM.

It is important to note that only 0·2–0·3 ml. portions of solution were used, so that in the case of solution (*b*) 20–30 mM-Na came from the tissue: muscles treated in large portions of the 'high K' medium, in which the Na concentration remained low, did not recover their fresh weight and excitability in Na saline as well as those loaded in a solution having some Na present.

The muscles were loaded for a measured time and then lightly blotted. The tracer output was followed by passing them through a series of small volumes (often 1 ml.) of solution with measured times of immersion in each. The quantity of tracer collected in each of the small volumes was estimated by evaporating a portion to dryness in a planchette under an infra-red lamp. The radioactivities were then measured under a thin-window counter tube. Controls were made by evaporating small portions of the used loading solution made up to the standard volume by addition of the same solution as had been used in the experiment. The observed radioactivity depends slightly on the nature of the solution, because the density of the salt deposit affects the self-absorption of the radiation by the sample. When the washing-out solution contained a high proportion of K a slightly increased blank (amounting to 4 counts/min) was introduced by the natural radioactivity of ^{40}K.

To improve the reproducibility of the readings the planchettes to be used were first treated with water-repellent silicone round their edges. To reduce dampness during counting the dried samples were kept on a hot plate during the time that individual planchettes were being moved into the counting apparatus. Counts were made for a number of 1 min periods but these were spaced out so that the planchette had been re-dried before each essay.

RESULTS

The results of the tracer output measurements will be given in subsections dealing separately with Cl^- output from muscles loaded in KCl, and with Cl^- output from muscles loaded in NaCl mixture, and Br^- output from muscles loaded in NaBr mixture. The reasons for this diversity of treatment are that an adequate quantity of Cl^- tracer is most easily introduced into the tissue by use of the KCl medium, but as the results obtained with chloride showed certain differences it was thought desirable to confirm them with the more radioactive Br preparations.

Cl output from muscles loaded in KCl

When muscles were loaded by immersion in 120 mM-KCl mixture (see Methods, *b*) for about 1 hr they swelled to about 1·5 times their original weight. The amount of KCl gained was 40–60 m-equiv/kg of *swollen* weight. If an experiment was carried through in such a way that the muscles reverted to their original weight (as they did in all the Na solutions and several of the K solutions tested) they could then be reloaded and used again if desired. If the muscles were exposed to NaCl, NaBr or $NaNO_3$ media they regained excitability. The initial part of the loss of the tracer from the loaded muscle includes diffusion from the extracellular space. The results showed that by working at 0° C it was possible to distinguish a rapid fraction and a slower-moving fraction. The temperature coefficient of the extracellular diffusion process is low compared with that of exchange of the cellular Cl^-. If the experiments in Cl^- media had been started at 20° it would not have been possible to distinguish between extracellular and cellular fractions (this difficulty is mentioned by Levi & Ussing, 1948). At the beginning of experiments like those shown in Figs. 1 and 2 the amount of the fast fraction fell to half in about 1 min. This half-time agrees with the value found for extracellular K^+ ions at 18° C diffusing from a muscle loaded in potassium phosphate (Harris, 1952). The quantity of Cl^- associated with the rapid fraction was such as to correspond to an extracellular space of between 13 and 40%. The variability is partly due to the muscles not having been blotted before use; a similar range has been reported by Johnson (1955) using sulphate, sucrose or the fast-moving Na fraction to measure the space.

It is not excluded that part at least of the fast fraction may properly belong to the intracellular Cl^-. If movement of Cl^- is limited by diffusion within the cells as well as by the resistive membrane, the Cl^- output will not obey a simple exponential law; instead it will have a time relation given by the sum of a series of exponential terms of which eventually only the first is important.

Table 1. Rate constants for output of Cl^- at 0° C and 20° C in various media

The rate constants have been calculated by dividing the amount of tracer Cl^- emerging in a given time by the product of the time interval (min) and the geometric mean of the tissue contents of tracer Cl^- at the beginning and end of the period. The first period used for the 0° C series covers the interval 9–14 min. The first period at 20° C was only 3 min in duration and has not been used for calculation because there may have been a time lag and in some runs a weighing was interposed. The standard deviation due to counting and timing errors is ±0·0015 in the 0° C values, and ±0·003 in the first three 20° C values rising to ±0·005 for the last value. The muscles had been loaded in KCl mixture, so that output takes place along with shrinkage

Solution	Successive rate-constant values (min^{-1})		Ratio of means 20:0° C
	At 0° C	At 20° C	
Chloride mixture composition same as loading solution (*a*)	0·031, 0·030, 0·029, 0·033	0·085, 0·087, 0·091, 0·082	2·8
	0·029, 0·027, 0·027, 0·031	0·084, 0·088, 0·084, 0·094	3·1
Br^- mixture with 90 mM-Br^- replacing Cl^-	0·014, 0·013, 0·011, 0·011	0·027, 0·036, 0·038, 0·043	3·2
Br^- mixture with 45 mM-Br^- replacing Cl^-	0·016, 0·016, 0·016	0·060, 0·052, 0·050, 0·044	3·1
CNS^- mixture with 5·5 mM-CNS^- replacing Cl^-	0·017, 0·014, 0·012, 0·011	0·033, 0·036, 0·040, 0·032	2·9
NO_3^- mixture, with 45 mM-NO_3^- replacing Cl^-	0·019, 0·015, 0·012	0·055, 0·040, 0·043, 0·041	—
I^- mixture with 60 mM-I^- replacing Cl^-	0·012, 0·007, 0·004, 0·003	0·012, 0·014, 0·014, 0·014	—
I^- mixture with 30 mM-I^- replacing Cl^-	0·012, 0·006, 0·005, 0·004	0·017, 0·020, 0·025, 0·022	—
CNS^- mixture with 22·5 mM-CNS^- replacing Cl	0·022, 0·013, 0·009	0·044, 0·029, 0·025, 0·028	—

The early, more rapid, part of the diffusion process may be partly confused with movement of the true extracellular material. Later in this paper it is argued that when Cl^- movement has been slowed by application of foreign anions the residual movement is of Cl^- along with K^+. It has already been shown that movement of cell K^+ has a time course which fits a combined diffusion–permeation process (Harris, 1957) and one might expect Cl^- movement into solutions of foreign anions to behave similarly. There is some indication that this is the case; particularly when movement had been slowed down it was not possible to establish a constant value for an exponential rate constant at 0° C within the 30 or 40 min given at this temperature (Table 1). Using Cl^- or Br^- solutions the Cl^- movement gave no evidence of being limited by diffusion; that is, a steady rate constant was attained within the 10 min required to ensure clearance of the extracellular material. In this case it was possible to make an estimate of the Q_{10} of the exponential output, which came to 1·7. Table 1 gives the ratio of the rates for a 20° C temperature interval.

Comparisons of the output to different solutions were made either by using paired muscles (from the same frog) loaded together and then exposed respectively to NaCl mixture of composition similar to that of the loading solution (*a*) (see Methods), and to the test solution with part or all of the Cl^- replaced by foreign anion (Fig. 1 *A*); or alternatively the exposures to different solutions were made in series during collection of the output from a single specimen (Fig. 1 *B*). The time for the amount remaining in the muscle to fall to 1/*e* of the level at a selected zero time was measured from the graph and used to obtain the rate constants in Table 2. It is more accurate to calculate the rate for each interval over which measurement was made (as in Table 1) but for most comparisons the graphical method is sufficiently close.

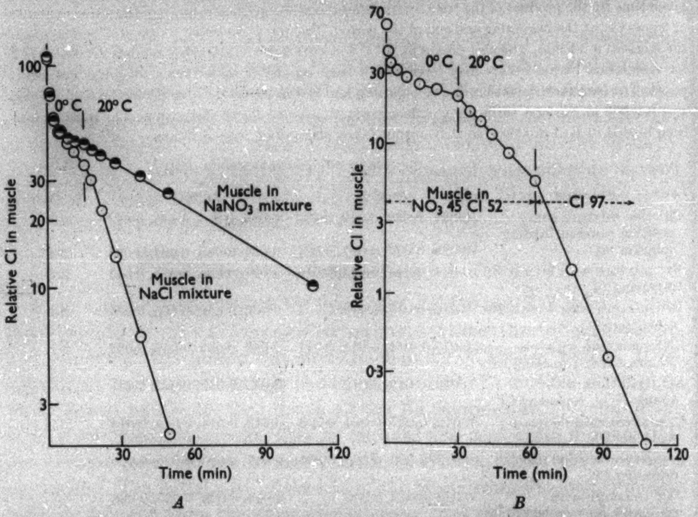

Fig. 1. *A*. Comparison of the Cl^- output from paired muscles which had been loaded in KCl and then exposed either to saline mixture with Cl^- (97 mM) or NO_3^- (90 mM) + Cl^- (7 mM). The first 15 min was at 0°. The relative amounts of tracer plotted are close to the contents (in μequiv/g) of initial swollen weight. *B*. Comparison of the Cl^- output from a KCl loaded muscle immersed first in 45 mM NO_3^- + 52 mM Cl^- mixture and later in 97 mM Cl^- solution. The first 30 min was at 0°.

Table 2 *A* shows that Cl leaves the muscle more slowly when foreign anions are substituted for Cl in the solution. Replacement of only a part (1/9 by CNS

TABLE 2. Rate constants for Cl^- output measured from graphs of content against time

Comparisons are made between different solutions using either paired muscles (Method *A*) or one muscle successively in test solution and then in Cl^- mixture (Method *B*). All solutions had Na^+, 120; K^+, 3; Ca^{2+}, 2 mM (or saturated if this was less than 2 mM).

Section *A*. Muscles loaded in KCl mixture so that output takes place along with shrinkage.

		Muscle in normal solution with 97 mM-Cl^-, 30 mM-HCO_3^-.		Muscle in solution with part Cl^- replaced by foreign anion		
	Temp. (° C)	Rate constant (min^{-1})	Method	Concn. of test anion (mM)	Rate constant (min^{-1})	Ratio of rates of Cl^- output
i	20	0·087	*A*	90 Br^-	0·038	2·1
ii	19	0·11	*B*	45 Br^-	0·059	1·9
iii	22	0·077	*A*	90 NO_3^-	0·015	5·0
iv	19	0·091*	*B*	45 NO_3^-	0·040	2·3
v	20	0·087	*B*	60 I^-	0·012	6·8
vi	20	0·087†	*A*	30 I^-	0·023	3·7
vii	22	0·077	*A*	45 I^-	0·019	4·0
viii	20	0·11‡	*A*	22·5 I^-	0·053	2·1
ix	20	0·083§	*A*	22·5 CNS^-	0·026	3·1
x	23	0·091	*A*	11 CNS^-	0·030	3·0
xi	20	0·095	*A*	5·5 CNS^-	0·036	2·6
xii	19	0·10	*B*	127 HCO_3^- with 5% CO_2 + 95% O_2	0·029	3·4
xiii	20	0·10‖	*B*	127 HCO_3^- with 100% CO_2	0·018	5·6
xiv	20	0·063	*B*	90 ClO_4^-	0·016	5·4
xv	20	0·063	*B*	45 ClO_4^-	0·016	5·4
xvi	20	0·072	*B*	5·5 ClO_4^-	0·045	1·6

* Pair of ii; † pair of v; ‡ no. ii used again; § no. iv used again; ‖ pair of xii.

Section *B*. Muscles loaded in NaCl mixture so that output takes place with little or no change in volume. The output does not follow an exponential law so that rate has been measured at the initial part of the curve just after raising the temperature from 0 to 20° C. The quoted value holds for more than half the total quantity of internal Cl^-.

		Muscle in normal solution with 97 mM-Cl^-, 30 mM-HCO_3^-.		Muscle in solution with part Cl^- replaced by foreign anion		
	Temp. (° C)	Rate constant (min^{-1})	Method	Concn. of test anion (mM)	Rate constant (min^{-1})	Ratio of rates of Cl^- output
xvii	20	0·12	*A*	90 Br^-	0·05	2·5
xviii	21	0·10	*A*	90 NO_3^-	0·043	2·3
xix	21	0·10	*A*	45 I^-	0·047	2·1
xx	19	0·063	*B*	22·5 CNS^-	0·02	3·1

Section *C*. Muscles loaded in NaBr mixture (as solution (*a*) in Methods but with 90 mM-Br^- substituted for Cl^-). The output does not follow the exponential law (cf. section *B* of this table) so the rate holding just after the temperature change has been given. Note that values in this section are for Br^- output.

		Muscle in normal solution with 97 mM-Cl^-, 30 mM-HCO_3^-.		Muscle in solution with part Cl^- replaced by foreign anion		
	Temp. (° C)	Rate constant (min^{-1})	Method	Concn. of test anion (mM)	Rate constant (min^{-1})	Ratio of rates of Cl^- output
xxi	19	0·077	*A*	90 Br^-	0·052	1·4
xxii	20	0·083	*A*	90 NO_3^-	0·047	1·75
xxiii	20	0·10	*A*	45 I^-	0·038	2·6

or 1/3 by I) of the Cl can lead to output becoming three times slower than in Cl. The rate is restored to the original value by changing the solution to Cl mixture. If the muscles were reloaded at the end of a run, the output to Cl mixture was at the normal rate (time constant 10–13 min), even if another solution had been applied during the previous output.

In the course of this series of experiments the muscles were weighed at intervals. It was found that at 0° C they did not revert from their initial swollen condition perceptibly in any solution. After the change to 20° C they lost weight in all the solutions of Na salts, and in solutions of K_2SO_4, $KHCO_3$ and $KH_2PO_4 + K_2HPO_4$, so that they reverted to their original weight in 1–2 hr, the time varying according to the initial amount of swelling.

Muscles loaded in the KCl medium were used to compare rates of Cl output to solutions of the K and Na salts of some anions. To the Na salt solution, 3 mM of the K salt was added and to each solution a trace of CaO (11 mg CaO/100 ml.). The results of the K–Na salt comparisons are collected in Table 3. In several mixtures the rate is not constant; these are noted in the table. When the anion is bicarbonate, phosphate or sulphate the output of Cl takes place for an hour at equal rates into Na and K salts (Fig. 2). Weight changes are similar in each. It is notable that movement is slower into bicarbonate with pure CO_2 than when CO_2/O_2 is used (Table 2, xii, and Table 3, v and vi). With the penetrating anions Cl and NO_3 the output to the K salt is slower than that to the Na salt (Table 3); at the same time the muscle tends to gain weight in the K solution and to lose it in the Na solution.

^{36}Cl and ^{82}Br output from muscles loaded in solutions of the respective Na salts

When muscles are loaded in the ordinary saline mixture there is little gain of Cl (or Br) by the cells and the tracer output from these muscles is derived mainly from ions present in the extracellular space. Later movement can, however, be followed and with the Cl and the more highly radioactive Br it was found that the simple logarithmic law did not hold. The rate appears to diminish with time (Fig. 3). This could be a consequence of the strong adsorption of a very little Cl^- or Br^- (about 1/1000 of the total anion content, including the extracellular part). An approximate 'rate constant' for the earlier part of the output of the cellular fraction is given in Table 2 *B*, *C*.

The results obtained with the muscles loaded in the Na mixtures show that much of the Cl^- output is at the same rate as that from muscles having an artificially raised Cl^- content from their exposure to KCl loading solution. Of interest is the observation that *output* of Br^- to a given solution takes place at a rate little less than that of Cl^- output. Fig. 3 shows experiments made with both tracer Cl^- and Br^--loaded paired muscles, in solutions with inactive Cl^- or Br^-. The counting rates have been scaled so that they are nearly equal at the time (15 min) of temperature change. It can be seen that Cl^- moves into

Cl^- very little quicker than Br^- moves into Cl^- and that Cl^- moves into Br^- very little quicker than Br^- moves into Br^-. The lines have comparable curvatures.

TABLE 3. Comparison of Cl^- output rates to salts of Na and K. Paired muscles were used; they were loaded in KCl mixture

	Temp. (° C)	Solution 1 composition (mM)	Rate constant (min^{-1})	Solution 2 composition (mM)	Rate constant (min^{-1})
Penetrating anions					
i	23	KCl 90 $KHCO_3$ 30 $CaCl_2$ 2	0·033 (becoming slower)	NaCl 90 $NaHCO_3$ 30 KCl 3 $CaCl_2$ 2	0·09
ii	20	KNO_3 90 $KHCO_3$ 30 $CaCl_2$ 2	0·012 (becoming slower)	$NaNO_3$ 90 $NaHCO_3$ 30 KNO_3 3 $CaCl_2$ 2	0·037
Non-penetrating anions					
iii	20	K_2HPO_4 50 KH_2PO_4 50 +CaO 11 mg/100 ml.	0·015	Na_2HPO_4 48·5 NaH_2PO_4 48·5 KH_2PO_4 1 KH_2PO_4 1 +CaO 11 mg/100 ml.	0·019
(Later in NaCl mixture both had rate constant 0·091 min^{-1})					
iv	22	K_2SO_4 83 $KHCO_3$ 17 +CaO 11 mg/100 ml.	0·024	Na_2SO_4 80·5 $NaHCO_3$ 16·5 K_2SO_4 1·5 +CaO 11 mg/100 ml.	0·024
(Later in NaCl mixture: 0·066 min^{-1} and 0·060 min^{-1}, respectively)					
v	23	$KHCO_3$ 120 +CaO 11 mg/100 ml. with 5% CO_2 +95% O_2	0·033	$NaHCO_3$ 117 $KHCO_3$ 3 +CaO 11 mg/100 ml. with 5% CO_2 +95% O_2	0·033
vi	18	with 100% CO_2	0·016	with 100% CO_2	0·016

Analytical experiments on NO_3^-, I^- and CNS^- outputs

In order to gain knowledge of whether those ions which impede Cl^- exchange do move more slowly than Cl^- in the tissue, experiments were made with muscles which had been treated with raised K^+ concentration in presence of the respective ion and Cl^-. The output of ion to the NaCl mixture (similar to Methods solution *a*) was found analytically.

Nitrate. A set of muscles was loaded for 70 min in KNO_3 (90 mM) + $KHCO_3$ (30 mM) + $CaCl_2$ (2 mM). Output of the NO_3^- to the NaCl mixture had a short fast component corresponding to the expected extracellular fraction and a slow component amounting to 60 μequiv/g swollen wt., which did not follow an exponential law. The time 'constant' was 80 min at 30–60 min, rising to 100 min or more by 90 min. The amount of NO_3^- lost in the first hour was 22 μequiv/g swollen wt. The output can better be fitted by assuming that

NO_3^- movement is following a diffusion law similar to that followed by K (Harris, 1957). The output could be entirely provided by NO_3^- emerging with K^+, and there is no evidence that exchange of Cl^- for NO_3^- took place. After exposure to the washing solution during the night excitability was regained.

Iodide. A set of muscles was loaded 70 min in KI (60 mM) + NaCl (45 mM) + $NaCO_3$ (15 mM) + $CaCl_2$ (2 mM). The output of I^- to NaCl mixture had a fast component corresponding to the expected extracellular fraction and a slower fraction which did not follow the exponential law indefinitely. Between 15 and 120 min the time constant was about 60 min, but a residual amount of I^- (equivalent to 1 μequiv/g swollen wt.) did not diminish further in 1 hr. The

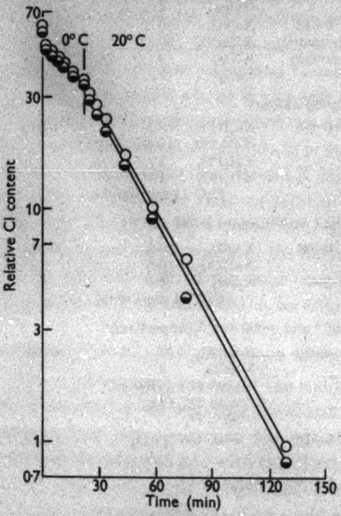

Fig. 2

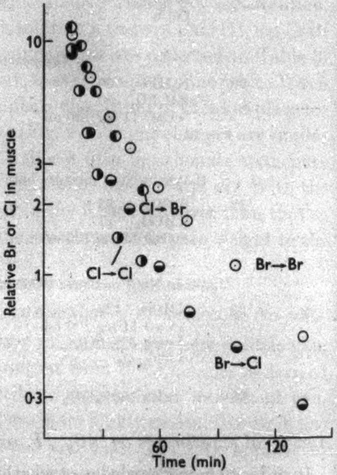

Fig. 3

Fig. 2. The output of Cl^- from paired muscles loaded in KCl mixture and then immersed in (○) 0·12M-$NaHCO_3$ or (◒) 0·12M-$KHCO_3$, with 5% CO_2 + 95% O_2 mixture passed into the solutions. The rate of Cl^- output is nearly the same and reversion of weights of the initially swollen muscles to the fresh weight took a similar course in each solution.

Fig. 3. Comparisons of the outputs of Cl^- and of Br^- to Cl^- and Br^- solutions, using muscles loaded in NaCl or NaBr media. The Cl^- output from individuals of a pair loaded in NaCl was observed in Cl^- and Br^- solution (Cl→Cl and Cl→Br points) and the Br^- output from individuals of a different pair loaded in NaBr was observed in Cl^- and Br^- solutions (Br→Cl and Br→Br points). The readings of radioactivity have been scaled to be nearly the same at 15 min (the time at which the temperature was raised from 0° to 20° C). The curvature seen in these plots was always present when NaCl or NaBr loading had been used, but the lowest-level of activity is only about 1/1000 of the initial activity (not shown on the graph). The estimated Cl content of the NaCl-loaded muscles was 8 μequiv/g.

time constant of the mobile part (15 μequiv/g swollen wt.) was 30 min. The muscles did not regain excitability (after 3 hr) and it is likely that the high internal I^- concentration has brought about an increased K^+ loss leading to I^- output being faster than if the cell had not been damaged.

Thiocyanate. A set of muscles were loaded 100 min in KCNS (24 mM) + NaCl (96 mM) + $NaHCO_3$ (24 mM) + $CaCl_2$ (2 mM). The output of CNS^- to NaCl mixture had a fast component corresponding to the extracellular fraction and a slower component amounting to about 7 μequiv/g swollen wt., with time constant 46 min. The CNS^- had nearly all left the muscles after 150 min but they remained in contracture and were opaque. As in the case of the KI-loaded muscles it is likely that the CNS^- loss was taking place along with K^+ rather than in exchange for Cl^-.

Stimulation

It was thought possible that when Cl^- output had been slowed by use of a foreign anion the remaining output would show sensitivity to stimulation. Before stimulation could be effective it was necessary to wait for excitability to return to the muscles, since these had been loaded in a KCl-NaCl mixture with 70 mM-KCl (and 2 mM-$CaCl_2$). The experiments were made as already described, but during some periods of output stimuli at 36 pulses/min were applied from platinum wires immersed in the portion of fluid. The rate constants were calculated as in Table 1 with the following results:

(*a*) Using 90 mM-NO_3 mixture at 20° C, rate constants (per minute) in successive periods were: 0·015, 0·015, 0·017, 0·019 (excitability just returned); stimulated, 0·028; resting, 0·021; stimulated 0·026; resting 0·020, 0·016.

(*b*) Using 60 mM-I mixture, rate constants were: 0·014, 0·014; stimulated, 0·019; resting, 0·014, 0·015; stimulated, 0·019; resting, 0·018, 0·014.

In each experiment the statistical errors give rise to a standard deviation of not more than ±0·001; it therefore appears that stimulation induces an increase of Cl^- output which may persist into the period following stimulation.

DISCUSSION

It is found that the rate constant for Cl^- output depends on the nature of the anion in the external solution; and that it is the same whether the muscles have been loaded in NaCl mixture without change of resting potential, or in KCl with consequent depolarization and net movement of K^+ and Cl^- ions (compare results in Table 2 *B* with those of 2 *A*). Movement of a single ion species would set up a large potential difference to oppose it. It will not be correct to think of independent movement of the respective ions; the Cl can only leave the cell either in company with equivalent cation or if it is replaced by anions drawn from the exterior. In solutions of non-penetrating anions (as phosphate, sulphate and bicarbonate appear to be) the slow Cl^- output must be determined by simultaneous $Na^+ + K^+$ output. The loss of KCl and water from the swollen KCl-loaded muscles can be inferred from the observation that the weights reverted to the fresh weights during exposure to the Na salt media. Actual measurement of K^+ output from paired loaded muscles to portions of K-free NaCl mixture or $NaNO_3$ mixture showed that it was about the same in each. Neglecting a rapid loss corresponding to extracellular K^+, lasting only 5 min, the K^+ loss in the first hour of an experiment made like the tracer runs with 15 min at 0° and 45 min at 20° was 20 μequiv/g initial swollen weight. The amount of Cl^- which left a KCl-loaded muscle either with K^+ or in exchange for NO_3^- was 29 μequiv in the first hour, while in a parallel experiment in Cl^- solution much more tracer had left the loaded muscle because of the rapid Cl^--for-Cl^- exchange. When only non-penetrating anion is present the Cl^- lost in the first hour is within the amount set by K^+ output; from a muscle with 40 μequiv/g swollen wt. of extra Cl^-, the loss was 22 μequiv Cl^- in the first hour to $NaH_2PO_4 + Na_2PO_4$ solution and from one loaded with 21 μequiv extra Cl^- the loss was 11 μequiv Cl^-. While it is reasonable to accept that Cl^- output from KCl-loaded muscles is not less than the equivalent K^+ output, and it can be more if exchangeable anion is present, the question arises as to what moves with the Cl^- in normal muscles without artificially raised K^+ content, such as the NaCl-loaded muscles. These have low Cl^- contents; the values found were 3–10 μequiv/g. Such small quantities could be accompanied either by cellular Na^+ or K^+, in addition to being able to exchange for external anion.

The results cannot be fitted on the single assumption that some anions replace internal Cl^- more slowly than does the external Cl^-, because the greatest effect would then be given by non-penetrants; in mixtures, Cl^- output would be at a rate not less than such fraction of the original Cl^- as remained times the normal rate. In fact, replacement of half the Cl^- by I^- causes the rate to fall to one-quarter and replacement of only 1/9 by CNS^- causes the rate to fall to one-third. There is evidently preferential concentration of I^- and still more of ClO_4^- and CNS^- in a region where Cl^- exchange can be impeded. It is notable that the effect is reversed within a time consistent with extracellular equilibration and that it is not progessive; the rate constant in a $CNS^- + Cl^-$ mixture is steady, it does not diminish (provided the KCl-loaded muscles are used). This points to the controlling action of I^- and CNS^- being exerted in a thin region, perhaps the membrane, which rapidly equilibrates with the external solution. The fact that mixtures of half NO_3^-, or Br^-, with half Cl^- show rates which are less than those calculated for the mean of output rates to Cl^- alone and the other anion alone, indicates that these ions are also concentrated to some extent in the controlling region, but Br^- more than NO_3^-. The relative order of degree of concentration appears to be $CNS^- = ClO_4^- > I^- > Br^- > NO_3^-$, this being the lyotropic series which is said to correspond to the adsorbability of the ions (Weiser, 1949). It is noteworthy that the effect of an ion on the rate of Cl^- output will, on this second necessary assumption, depend both on its own internal mobility (which NO_3^-, Br^-, I^- and CNS^- have been shown to possess) and on the degree to which it is concentrated in the controlling region. A further effect can, however, arise if the foreign anion increases the rate at which the cell loses K^+. We have seen that the minimum rate of Cl^- loss is likely to be set by the rate of K^+ loss; if a poisonous substance increases K^+ loss it will also increase the minimum rate of Cl^- output. It has been shown that CNS^- and sometimes I^- produce an increased rate of K^+ output (Edwards *et al.* 1957). Hence, even if sufficient CNS^- is mixed with Cl^- to inhibit completely Cl^--for-Cl^- exchange, the Cl^- output may be faster than that seen when using a less poisonous anion (as ClO_4^- may be), because CNS^- causes an accelerated rate of KCl escape. This may explain why the CNS^- effect remains at a slowing by a threefold factor while NO_3^-, ClO_4^- and non-penetrants give larger factors. The dual influence of CNS^- has also been seen by Padsha (private communication) who finds that resistance first increases and then falls as the concentration is raised. The slowing of Cl^- movement by ions ranking higher in the lyotropic series may be compared with the inhibition of iodide accumulation in a seaweed by CNS^- and ClO_4^- (Klemperer, 1957) and with the interference by ClO_4^- with iodide secretion into saliva (Burgen & Seeman, 1957).

The observation (Table 3i and ii) that Cl^- output is slowed in solutions of K^+ salts of the penetrating ions Cl^- and NO_3^- may be related to a mutual interference between ions seeking to emerge and the inward stream of K^+ salt which is in progress.

The interchange of Cl^- for a less mobile anion will set up a diffusion potential and it is at first sight surprising that the measured resting potential is nearly insensitive to the nature of the external anion when Br^- or NO_3^- are applied (Harris & Martins-Ferreira, 1955) and in I^- and CNS^- solutions (Lubin, 1957). However, the resting potential is the potential difference between the inner K-rich region of the cell and the solution. It may be made up of two components, a Donnan or diffusion potential between the solution and an outer non-selective region of the cell (discussed by Edwards & Harris, 1957 and Harris, 1957) and that between inner and outer regions (Fig. 4, i). The outer region mentioned here is not identified with the site of control of Cl^- movement by the other anions; this site may be membrane shown by the double line on Fig. 4. A diffusion potential set up by putting a muscle into, say, nitrate solution will shift the outer potential difference and lead to a change in the ionic composition of the outer region (Fig. 4, ii). The sum of the two p.d. will return to a value near to that given by the ratio of the K^+ concentrations in the inner region and the solution after a transient positive phase. The transient will last only while the K^+ concentration in the outer region falls to a new value (Fig. 4, iii) determined by the relative mobilities of the ions moving between solution and outer region. Thereafter, the concentrations in the outer region will shift and eventually the diffusion potential will disappear and the local K^+ concentration in the outer region be restored. With a whole muscle the extracellular diffusion makes observation of these effects difficult, but a transient positivity has been described by Hodgkin & Horowicz (1957) when a single KCl-loaded fibre is immersed in sulphate solution.

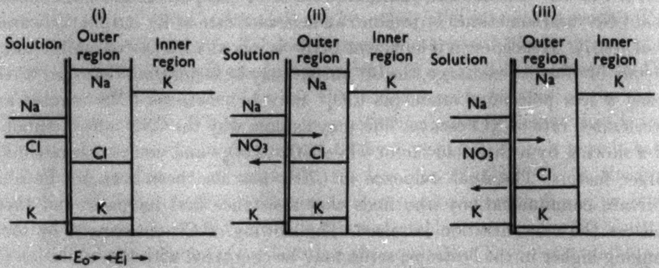

Fig. 4. (i) Diagram showing concentration levels of Na^+, Cl^- and K^+ in the solution and in the two regions of a resting muscle. The measured resting potential is the sum of an inner p.d. E_i between the selective K-permeable region and the non-selective 'outer region' given by $RT/F \ln K_i^+/K_r^+$ where K_i^+ and K_r^+ are the respective concentrations and the outer p.d. E_o across the membrane which is determined by the concentration of fixed charges in the outer region (it is here a Donnan potential). The measured p.d. is the sum of E_i and E_o. (ii) Diagram showing concentration levels at the instant the muscle from (i) is put in NO_3^- mixture, and the directions of net ion movements (shown by arrows). The K_r level has not had time to alter so E_i is unchanged but E_o is reduced and perhaps changed in sign by the unequal mobilities of the NO_3^- and Cl^-. As a result the level K_r tends to fall. The sum of E_i and E_o will be less than in (i). (iii) Diagram showing the condition later in the exposure to NO_3. Sufficient K has emerged from the outer region to restore electrochemical balance between the outer region and the solution; since K_r is now lower, the value of E_i will be increased and the over-all p.d. is close to $RT/F \ln K_i^+/K_s^+$ as it was in (i).

The results can be related to two sets of observations of electrical phenomena. The rate of return of the resting potential after a subthreshold stimulus or after elicitation of an end-plate potential is reduced by the same conditions which lead to slower Cl^- output, that is by the anions Br^-, NO_3^-, I^- and SO_4^{2-} (Padsha, 1957). Similarly, after an action potential the later part of the repolarization is made slower by the foreign anions. This has been described by Etzensperger & Bretonneau (1956) for I^-, Br^- and NO_3^- and by Lubin (1957) for I^- and CNS^- (though not for NO_3^-). Some records obtained by Dr C. Edwards in Br^-, NO_3^- and I^- solutions, and during subsequent washing in normal Cl^- mixture, are reproduced in Fig. 5. During exposure (left-hand column of records) there is a long tail or after-potential which is absent after 30 min washing in Cl^- mixture. From these observations it can be inferred that Cl^- movement plays a large part in readjustment of the potential difference following a disturbance. Since an action potential involves an outward movement of K^+, it would be expected that in circumstances where most of the Cl^- output takes place along with K^+ rather than in exchange for external anion stimulation would lead to increased Cl^- output, as was indicated in the two experiments described. It is proposed that return to the normal resting potential after an action potential depends on the time course of the re-establishment of the outer potential difference (E_0 in Fig. 4, i) and that it is here that inhibition of Cl^- movement exerts its influence.

In view of the strong effect of ClO_4^- on Cl^- movement, some experiments were made by the physiology course students of this College using the apparatus described by Ritchie (1954) to measure the duration of the 'active state'. It was found that replacement of only 5 mM of Cl^- by ClO_4^- prolonged the 'active state' as much as fully substituting NO_3^- for the Cl^-.

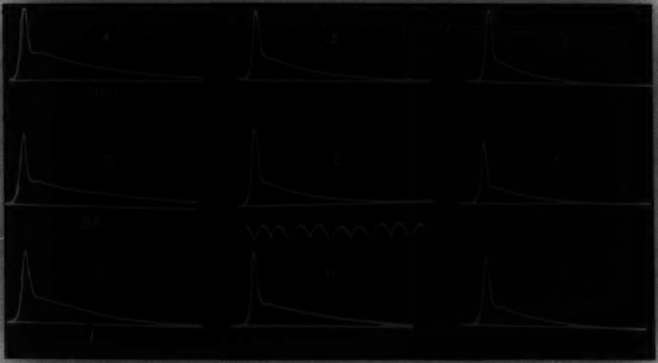

Fig. 5. Examples of records in a series obtained with external electrodes (one with high K^+) applied to a sartorius muscle which was stimulated in solutions with various anions. The records were taken in the order shown:

A after 12 min in 90 mM-NO_3^- mixture
  B in Cl^- mixture for 15 min following 30 min in NO_3^-
  C in Cl^- mixture for 30 min following 30 min in NO_3^-;
D after 12 min in 90 mM-Br^- mixture
  E in Cl^- mixture for 15 min following 30 min in Br^-
  F in Cl^- mixture for 35 min following 30 min in Br^-;
G after 8 min in 45 mM-I^- mixture
  H in Cl^- mixture 13 min following 30 min in I^-
  J in Cl^- mixture 26 min following 30 min in I^-.

It can be seen that only the later part of the return to full resting potential is slowed by the anions. The effect is reversed in between 15 and 30 min exposure to Cl^- mixture. On *E* a portion of two nearly out-of-phase 50 c/s wave forms provide a time scale.

SUMMARY

1. The output of tracer Cl^- from frog sartorii which had first been loaded in either KCl or NaCl mixtures was studied.

2. The output is slowed in solutions in which a proportion of the Cl^- has been replaced by Br^-, NO_3^-, I^-, ClO_4^- or CNS^- in order of increasing effect.

3. The output is slowed in solutions of non-penetrating anions (sulphate, phosphate, bicarbonate) and the K^+ and Na^+ salts of these anions given similar results.

4. The output is slower to KCl and KNO_3 solutions than to the respective Na^+ salt solutions.

5. Results with KCl- and NaCl-loaded muscles are similar in time scale, but differ in that after KCl-loading the output follows an exponential law, whereas after NaCl-loading output takes place at a diminishing rate.

6. Output of Br^- from muscles loaded in NaBr mixture (using tracer Br) behaves like Cl^- output from the NaCl-loaded specimens.

7. The observations are interpreted on the basis of Cl^- output being the sum of an exchange for penetrating anions, when these are present, and a net output of cation plus Cl^-. The slowing effect of I^-, ClO_4^- and CNS^- is greater than corresponds to their having zero mobility; they appear to be concentrated in a region where they impede Cl^- movement. As it was shown that I^- and CNS^- can enter and leave the tissue, their action on Cl^- exchange is the more striking.

I have to thank Dr D. Wilkie for supervising the measurements of the duration of the contraction in ClO_4^- solutions, and Dr C. Edwards for providing the records of Fig. 5. Part of the expenses of this work were met from a grant from the Government Grants Committee of the Royal Society.

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THE EFFECT OF CHANGES IN ION CONCENTRATION ON VENTRICULAR FIBRILLATION INDUCED ELECTRICALLY

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The experimental study of ventricular fibrillation presents difficulties when it is undertaken in the whole animal because fibrillation is usually a terminal event; the ventricular output falls to zero and as a result the coronary flow stops and the heart dies. It is, however, possible to make observations when the isolated heart of the rabbit is perfused through a cannula tied in the aorta. If fibrillation occurs the perfusion is not interrupted and therefore the heart continues to receive oxygen. While the fibrillation proceeds, a switch can be made to a different perfusion fluid in order to observe whether the change restores a normal rhythm. Thus factors which initiate and terminate fibrillation can be examined. Observations have been made in this way by Grumbach, Howard & Merrill (1954), by Dirken, Gevers, Heemstra & Huizing (1955) and by Armitage, Burn & Gunning (1957*a*, *b*). Grumbach and his colleagues made observations on potassium and calcium. They found that when they perfused the heart with a solution which was without potassium, ventricular fibrillation occurred spontaneously; this fibrillation was arrested and a normal rhythm restored when the perfusion was continued with a fluid containing potassium. Furthermore, they observed that perfusion of a solution without potassium did not cause fibrillation if the solution was also without calcium. The injection of calcium chloride into the cannula leading to the heart caused fibrillation to begin. If the solution contained a little potassium, namely 1·1 mM, spontaneous fibrillation occurred if the calcium was raised from a normal figure of 5·6 to 10·2 mM, but if the amount of potassium was greater than 2·3 mM then even so much as 30·5 mM calcium failed to induce spontaneous fibrillation.

Dirken and his colleagues produced fibrillation by stimulating the ventricular muscle electrically. This procedure was also used by Armitage *et al.* (1957*a*), who made a quantitative study of the effects of changes in the concentration of potassium and calcium in the perfusing solution. They were able to do this by observing the proportion of hearts which fibrillated when a solution of a given composition was used for perfusion. Thus in one heart the fibrillation produced by electrical stimulation might continue only for a minute or two after the stimulation stopped, a normal rhythm being restored; in another heart the fibrillation might continue indefinitely. They described the first heart as not fibrillating and the second as fibrillating. They observed that the proportion of hearts in which fibrillation persisted rose from 0 to 100% as the potassium in the solution was reduced from 11·2 to 1·4 mM. When the potassium was 11·2 mM, which is twice the normal concentration, electrical stimulation failed to produce fibrillation, whereas at the other extreme, when the potassium was 1·4 mM most hearts fibrillated spontaneously before electrical stimulation was applied. While these results with different concentrations of potassium were straightforward, results with different concentrations of calcium were not, and the graph relating the concentration of calcium to the proportion of hearts fibrillating contained two peaks. The graph could not be interpreted, and fresh observations have accordingly been made which are described in this paper. It has been found that the proportion of hearts fibrillating in a given concentration of calcium depends on the concentration of potassium, and this relation has been investigated as fully as possible. The effects of changes in the concentration of sodium and of chloride and of introducing magnesium have also been studied.

METHODS

The method has been similar to that described by Armitage *et al.* (1957 *a*). Hearts freshly excised from rabbits were perfused through the coronary vessels with fluid kept at constant temperature (Saxby, 1956). A mechanical record was obtained on a smoked drum by a thread attached to a pin in the apex of the ventricle. One platinum electrode was inserted in the base of the ventricles and a second was attached to the apex. Through these electrodes stimulation was applied, and at other times the electrodes were used as leads to a Cossor electrocardiograph (Model 1314). The stimuli were square wave pulses of strength 1 mA and duration 0·75 msec, and were applied at a rate of 700/min for 5 min. When stimulation stopped each heart was observed to see if it reverted to normal rhythm. Hearts reverting within 15 min were recorded as 'not fibrillating', hearts not reverting within this time as 'fibrillating'; reversion usually occurred within 5 min. In all experiments the effect of perfusing different solutions was tested. Thus if it was found that persistent fibrillation followed stimulation when the perfusion fluid contained a normal concentration of potassium and calcium, the fibrillation was arrested by pouring cold saline over the outside of the heart (Dirken *et al.* 1955). Perfusion was then continued with a solution of different composition. In the course of one experiment a heart was sometimes perfused with eight different solutions, and stimulation was applied to determine their effect. The normal perfusion fluid contained (mM) NaCl 132, KCl 5·6, $CaCl_2$ 2·2, NaH_2PO_4 0·92, $NaHCO_3$ 25, glucose 11·1, sucrose 13·1. The solution was thoroughly saturated with O_2 95% + CO_2 5% (McEwen, 1956). To replace half the chloride by sulphate, a given volume of this solution was mixed with an equal volume of a solution in which 65 mM-Na_2SO_4 and 65·5 mM sucrose were substituted for 132 mM-NaCl.

RESULTS

Opposed effect of potassium and calcium

Some experiments were carried out to demonstrate the effect of changes in the potassium (K^+) and calcium (Ca^{2+}) concentrations in each heart. Results of four experiments are set out in Table 1, in which the concentrations of K^+ and of Ca^{2+} are stated in terms of the normal concentration. Thus when the concentration of K^+ is given as 1, this indicates a concentration of 5·6 mM, and a concentration of $Ca^{2+} = 1$ indicates a concentration of 2·2 mM.

The simplest experiment to follow is Expt. 3 in Table 1, in which stimulation did not produce fibrillation when the heart was perfused with a normal solution. The perfusion fluid was then changed to one in which the concentration of K^+ was reduced to 0·75 normal, and stimulation then caused fibrillation. At the end of 15 min this fibrillation was arrested by cooling the heart, and perfusion was continued with a solution containing $[K^+]$ 0·75 normal, but $[Ca^{2+}]$ 0·5 normal. On stimulation there was no fibrillation. However, when a new solution containing $[K^+]$ 0·5 normal and $[Ca^{2+}]$ 0·5 normal was perfused, stimulation once more caused fibrillation. Finally, stimulation during perfusion with $[K^+]$ 0·5 normal and $[Ca^{2+}]$ 0·25 normal did not cause fibrillation. Thus Expt. 3 showed (*a*) that a decrease in K^+ concentration alone without change in Ca^{2+} concentration led to stimulation producing fibrillation; (*b*) that the effect of the drop in K^+ concentration could be neutralized by a drop in Ca^{2+} concentration; and (*c*) that to neutralize the effect of a drop in the K^+ concentration the drop in the Ca^{2+} concentration had to be twice as great. This necessity for a greater drop in the Ca^{2+} concentration to neutralize a given drop in the K^+ concentration was also evident in Expt. 1. In this experiment fibrillation occurred when both $[K^+]$ and $[Ca^{2+}]$ were normal, but not when $[K^+]$ was raised to 1·5 normal. When $[K^+]$ was 0·75 normal fibrillation occurred, but when $[Ca^{2+}]$ was also reduced to 0·125 normal, stimulation did not cause fibrillation. $[Ca^{2+}]$ was then raised to 0·166 normal, and then to 0·25 normal,

TABLE 1. Effect of variations of $[K^+]$ and $[Ca^{2+}]$ in different hearts

When $[K^+] = 1$, it is 5·6 mM; when $[Ca^{2+}] = 1$, it is 2·2 mM

Expt. 1			Expt. 2			Expt. 3			Expt. 4		
$[K^+]$	$[Ca^{2+}]$	Result	$[K^+]$	$[Ca^{2+}]$	Result	$[K^+]$	$[Ca^{2+}]$	Result	$[K^+]$	$[Ca^{2+}]$	Result
1	1	+	1	1	+	1	1	−	1	1	+
1·5	1	−	1	0·83	+	0·75	1	+	1	0·75	−
0·75	1	+	1	0·75	+	0·75	0·5	−	0·75	0·75	+
0·75	0·125	−	1	0·5	−	0·5	0·5	+	0·75	0·5	−
0·75	0·166	−	0·75	0·5	+	0·5	0·25	−	0·5	0·5	+
0·75	0·25	+	0·75	0·25	−				0·5	0·25	−
0·5	0·125	−	0·5	0·25	+						
0·5	0·166	+	0·5	0·125	−						

+, fibrillation after stimulation; −, no fibrillation after stimulation.

and with this concentration there was again fibrillation. Hence to prevent the fibrillation occurring when $[\mathrm{K}^+]$ was 0·75 normal, $[\mathrm{Ca}^{2+}]$ had to be reduced to 0·166 normal.

In Expts. 2 and 4 a change was first made in the Ca^{2+} concentration; in Expt. 2 fibrillation was prevented by dropping the $[\mathrm{Ca}^{2+}]$ to 0·5 normal; in Expt. 4 it was prevented by dropping the $[\mathrm{Ca}^{2+}]$ to 0·75 normal. When the K^+ concentration was also reduced, stimulation again caused fibrillation. These experiments all demonstrated that a lowering of K^+ concentration favoured fibrillation while a lowering of Ca^{2+} concentration made it less likely.

Opposed effect of sodium and calcium

In the course of their observations, Armitage *et al.* (1957*b*) observed that a reduction in the concentration of sodium also favoured fibrillation. While the K^+ concentration and Ca^{2+} concentration could be modified without affecting tonicity, a reduction of NaCl concentration reduced tonicity. They compensated for this reduction by adding sucrose, but when this was done there was a decrease in chloride ions as well as in sodium ions. In other experiments they compensated for the reduction by adding choline chloride, so that sodium only was reduced; when this was done they gave injections of atropine at regular intervals to neutralize the effect of choline on the heart rate. They obtained the same results whether sucrose or choline chloride was used.

TABLE 2. Effect of changes in $[\mathrm{Na}^+]$ in relation to changes in $[\mathrm{Ca}^{2+}]$

When $[\mathrm{K}^+] = 1$, it is 5·6 mM; when $[\mathrm{Ca}^{2+}] = 1$, it is 2·2 mM; when $[\mathrm{NaCl}] = 1$, $[\mathrm{Na}^+]$ is 158 mM; when $[\mathrm{NaCl}] = 0·75$, $[\mathrm{Na}^+]$ is 125 mM; when $[\mathrm{NaCl}] = 0·5$, $[\mathrm{Na}^+]$ is 92 mM

Expt. 5				Expt. 6				Expt. 7				Expt. 8			
$[\mathrm{K}^+]$	$[\mathrm{Ca}^{2+}]$	[NaCl]	Result	$[\mathrm{K}^+]$	$[\mathrm{Ca}^{2+}]$	[NaCl]	Result	$[\mathrm{K}^+]$	$[\mathrm{Ca}^{2+}]$	[NaCl]	Result	$[\mathrm{K}^+]$	$[\mathrm{Ca}^{2+}]$	[NaCl]	Result
1	1	1	−	1	1	1	+	1	1	1	+	1	1	1	+
1	1	0·75	+	1	0·75	1	+	1·25	1	1	−	1	0·75	1	+
1	0·75	0·75	−	1	0·5	1	+	1·25	1	0·75	+	1	0·5	1	+
1	0·75	0·5	+	1	0·166	1	−	1·25	0·5	0·75	−	1	0·25	1	−
1	0·5	0·5	−	1	0·166	0·75	+					1	0·25	0·75	+
1	0·5	0·25	+									1	0·166	0·75	−

+, fibrillation after stimulation; −, no fibrillation after stimulation.

In the present experiments sucrose was added to maintain the tonicity, and the effect of changes in NaCl concentration was studied in relation to changes in Ca^{2+} concentration. The results of four experiments are given in Table 2. Since the concentration of Na^+ depended not only on the amount of NaCl but also on the amounts of $\mathrm{NaHCO_3}$ and $\mathrm{NaH_2PO_4}$, a reduction of [NaCl] to 0·75 normal meant that the Na^+ concentration fell from 158 to 125 mM, and a reduction of [NaCl] to 0·5 normal meant that the Na^+ concentration fell to 92 mM.

In Expt. 5 stimulation during perfusion with the normal solution did not cause fibrillation. When the [NaCl] was reduced to 0·75 normal, stimulation caused fibrillation. When this had continued for 15 min it was arrested by cooling the heart and the perfusion fluid was changed to one containing 0·75 normal Ca^{2+} concentration. Stimulation then did not cause fibrillation. The perfusion fluid was again changed to one containing [NaCl] 0·5 normal and again stimulation caused fibrillation. This alternation of lowering $[Na^+]$ with the result that stimulation caused fibrillation, and then of lowering $[Ca^{2+}]$ so that stimulation did not cause fibrillation, was continued down to a perfusion fluid containing Na^+ in a concentration of only 59 mM and Ca^{2+} in a concentration of 1·1 mM. Stimulation during perfusion with this solution caused fibrillation.

TABLE 3. Effect of changes in [NaCl] in relation to changes in $[K^+]$

When $[K^+]=1$, 5·6 mM; when $[Ca^{2+}]=1$, it is 2·2 mM; when $[NaCl]=1$, $[Na^+]$ is 158 mM; when $[NaCl]=0·75$, $[Na^+]$ is 125 mM; when $[NaCl]=0·5$, $[Na^+]$ is 92 mM

Expt.	K^+	NaCl	Result	K^+	NaCl	Result	K^+	NaCl	Result
9	1	1	–	1	0·5	+	1·25	0·5	–
10	1	1	–	1	0·5	+	1·5	0·5	–
11	1·5	1	–	1·5	0·5	+	2	0·5	–
12	1	1	–	1	0·5	+	2	0·5	–
13	1	1	–	1	0·5	+	1·5	0·5	–

+, persistent fibrillation; –, no fibrillation

In Expt. 6 the concentration of Ca^{2+} was changed first, and it had to be lowered to 0·166 normal before a perfusion fluid was found in which stimulation did not cause fibrillation. Lowering $[Na^+]$ to 0·75 normal led once more to fibrillation.

The experiments all showed that the fibrillatory effect of lowering the NaCl concentration could be counteracted by lowering the Ca^{2+} concentration. In this respect the effect of lowering the NaCl concentration was similar to the effect of lowering the K^+ concentration.

Relation of sodium to potassium

The relation of changes in NaCl to changes in K^+ was also studied and the results of five experiments are given in Table 3. The observations in Expt. 9 were typical of those in the others. When the heart was perfused with the normal solution, stimulation did not cause fibrillation. On reducing the NaCl to half the normal amount, so that $[Na^+]$ fell to 92 mM, stimulation caused fibrillation. When the $[K^+]$ was raised from 5·6 to 7 mM, the NaCl remaining unchanged, stimulation did not cause fibrillation. Thus the fibrillatory effect of low [NaCl] was compensated by raising the concentration of K^+.

Effect of reducing chloride ion concentration

Observations were also made to determine the effect of a fall in Cl^- concentration, by preparing solutions in which NaCl was replaced by Na_2SO_4 and sucrose. In these experiments the Na^+ concentration was unchanged. The results of ten experiments are given in Table 4. In each, concentrations of K^+

and of Ca^{2+} were determined in which stimulation did not cause fibrillation. The Cl^- concentration was then reduced to 0·5 normal, and in eight out of the ten experiments stimulation then caused fibrillation. It may be recalled that Armitage *et al.* (1957*b*) found that a reduction of [Na^+] effected by replacing NaCl by choline chloride caused fibrillation; it therefore appears that either a reduction in the concentration of Na^+ or a reduction in the concentration of Cl^- has a fibrillatory effect. By introducing 65·5 mM-SO_4^{2-}, the effective [Ca^{2+}] might have been reduced. However, a reduction of [Ca^{2+}] would have diminished the proportion of hearts fibrillating, and the result obtained was the reverse.

TABLE 4. Effect of reducing [Cl^-] to 0·5, replacing Cl^- by SO_4^{2-} and sucrose

Expt.	[K^+]	[Ca^{2+}]	Result	Result after [Cl^-] was reduced to 0·5
14	1	1	–	–
15	1	0·166	–	+
16	1·25	1	–	+
17	1·5	1	–	+
18	1·25	1	–	+
19	1·5	1	–	+
20	1·5	1	–	+
21	1·75	1	–	+
22	1·75	1	–	–
23	1·5	1	–	+

+, persistent fibrillation; –, no fibrillation

Action of Mg^{2+} in preventing fibrillation

The results at this point showed that a reduction in [K^+], or in [Na^+] or in [Cl^-] favoured fibrillation, while on the other hand a reduction in [Ca^{2+}] made fibrillation less likely. Alone of these ions [Ca^{2+}] had an effect favouring fibrillation.

Since Mg^{2+} ions neutralize the action of Ca^{2+} ions in various circumstances, observations were made to see whether Mg^{2+} would arrest ventricular fibrillation. In carrying out an experiment the ventricles were first stimulated during perfusion with a normal solution. If fibrillation resulted, the perfusion fluid was changed to one containing Mg^{2+} to observe whether the fibrillation was arrested. If fibrillation did not occur, either the K^+ content of the perfusing fluid was lowered or the Ca^{2+} content was raised, and the ventricles were stimulated again. When fibrillation occurred and was observed to continue for 15 min or more, the perfusion fluid was changed to one also containing Mg^{2+} to see if the fibrillation was arrested. It was found that fibrillation was arrested by Mg^{2+} provided the Ca^{2+} concentration was normal, the K^+ concentration being either normal or reduced. The action depended on the concentration of Mg^{2+} added as shown in Table 5; thus a concentration of 10 mM arrested fibrillation in one out of five hearts, while a concentration of

30 mM arrested fibrillation in five out of five hearts. However, when the Ca^{2+} concentration was doubled, the same concentration of Mg^{2+} failed to arrest fibrillation in three hearts, showing that the defibrillating action depended on an antagonism to Ca^{2+}.

Observations were also made to see the effect of Mg^{2+} in preventing the occurrence of fibrillation. Thus in the experiments already described when fibrillation was arrested by perfusion with a solution containing Mg^{2+}, the perfusion was then continued for 30 min with the same solution, and stimulation was applied. In those experiments in which the solution containing Mg^{2+} had not arrested fibrillation, a normal beat was restored by injecting KCl into the cannula, and the perfusion was continued for 20–30 min. Stimulation was then applied. Table 6 shows the effect of Mg^{2+} in preventing fibrillation. Those observations in which, in the absence of Mg^{2+}, fibrillation occurred when $[K^+]$ was normal, are shown separately according to the Ca^{2+} concentration. The last column includes those in the three middle columns together with those in which the $[K^+]$ was less than normal. The effect of Mg^{2+} in preventing fibrillation was less as the concentration of Ca^{2+} was higher.

TABLE 5. Effect of Mg^{2+} in arresting fibrillation after stimulation

Concentration (mM)		No. of hearts in which fibrillation was arrested	Mean time for arrest (min)
Mg^{2+}	Ca^{2+}		
10	2·2	1 out of 5	8
15	2·2	1 out of 2	5
20	2·2	3 out of 5	5
30	2·2	5 out of 5	3
30	4·4	0 out of 3	

TABLE 6. Proportion of hearts prevented by Mg^{2+} from fibrillating after stimulation. Observations in the three middle columns were made when $[K^+]$ was 5·6 mM

$[Mg^{2+}]$ (mM)	$[Ca^{2+}]$ (mM)			Proportion of all hearts
	2·2	4·4	8·8	
5	—	—	—	2 out of 4
10	1 out of 1	1 out of 1	0 out of 1	6 out of 9
20	4 out of 4	1 out of 2	0 out of 2	5 out of 8
30	4 out of 4	5 out of 6	2 out of 3	11 out of 13

Relation of Ca^{2+} to K^+

In their first paper Armitage *et al.* (1957 *a*) described the effect of changes in the Ca^{2+} concentration on the proportion of hearts fibrillating after stimulation. Their results were obtained in the presence of a normal concentration of K^+ and showed that when the concentration of Ca^{2+} was twice normal, stimulation caused fibrillation in all hearts. When the Ca^{2+} concentration was normal stimulation caused fibrillation in about 50% of hearts, and when the concentration was zero stimulation did not cause fibrillation in any heart. From this it would have been expected that as the Ca^{2+} concentration fell from normal to zero there would have been a gradual fall in the proportion of hearts fibrillating. Actually when the Ca^{2+} concentration was 0·275 mM (=0·125 normal), it was observed that stimulation caused fibrillation in 80% of hearts.

This finding seemed so improbable that it was decided to repeat the observations, and the results are given in Table 7. They did not confirm the previous result. They show that as the concentration of Ca^{2+} fell there was a gradual fall in the proportion of hearts fibrillating and there was no rise at a Ca^{2+} concentration of 0·275 mM. Table 7 also gives results obtained when the K^{+} concentration was 0·5 normal and 0·25 normal. The results are expressed graphically in Fig. 1. In addition to the above observations, however, others were made when the concentration of K^{+} was twice normal; in these experiments stimulation did not cause fibrillation in any heart whatever the Ca^{2+} concentration, even when it was raised to 8·8 mM, which was four times normal.

TABLE 7. Effect of varying [Ca^{2+}] on the proportion of hearts fibrillating

[Ca^{2+}]		[K^{+}] 5·6 mM		[K^{+}] 2·8 mM		[K^{+}] 1·4 mM	
(mM)	(normal)	No. of observations	(%)	No. of observations	(%)	No. of observations	(%)
4·4	2	12/12	100	—	—	—	—
2·2	1	15/28	53	12/13	95	10/10	100
1·1	0·5	7/15	47	8/9	90	4/4	100
0·55	0·25	3/8	37	5/10	50	5/9	55
0·275	0·125	6/21	30	5/15	33	3/13	23
0·1375	0·062	5/15	33	2/9	22	0/10	0

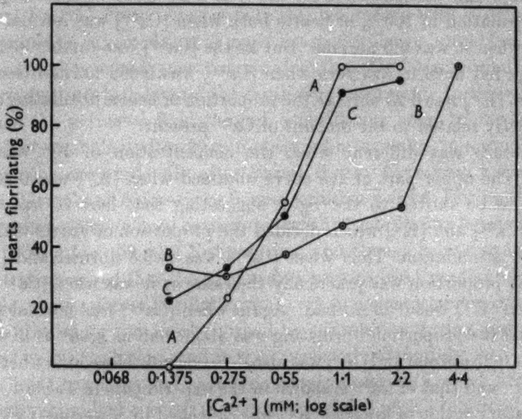

Fig. 1 Effect of [Ca^{2+}] on proportion of hearts fibrillating. *A*, [K^{+}] = 1·4 mM; *B*, [K^{+}] = 5·6 mM (normal); *C*, [K^{+}] = 2·8 mM.

DISCUSSION

As a first approximation to the facts it can be said that ventricular fibrillation occurs with more certainty as a consequence of electrical stimulation the lower the concentration in the perfusing fluid of K^+, of Na^+ and of Cl^- and the higher the concentration of Ca^{2+}. The clearest results were those obtained by Armitage *et al.* (1957*a*) with variations of K^+. Electrical stimulation never produced fibrillation when K^+ concentration was twice normal, and as the concentration fell the proportion of hearts in which electrical stimulation caused fibrillation steadily rose to 100% when K^+ concentration was 0·25 normal. This change might have been related to the increasing difficulty with which K^+ could be replaced in the cell by active transport, so that the net loss of K^+ was increased.

The effect of changes in the concentration of Ca^{2+} were more difficult to understand. They could, however, be followed very well in the present experiments when the concentration of K^+ was 0·25 normal. Stimulation then caused fibrillation in 100% of hearts both when $[Ca^{2+}]$ was normal (2·2 mM) and also when it was 0·5 normal. But as the $[Ca^{2+}]$ was further reduced the percentage fell until it was zero when $[Ca^{2+}]$ was 0·062 normal (see Fig. 1). Thus when $[K^+]$ was 0·25 normal, the proportion of hearts fibrillating appeared to be directly related to the amount of Ca^{2+} present.

The picture was different when the concentration of K^+ was normal (5·6 mM). The upper part of the curve obtained when $[K^+]$ was 0·25 normal appeared to be shifted to the right, suggesting that here it was the ratio between $[Ca^{2+}]$ and $[K^+]$ which decided the proportion of hearts which fibrillated after stimulation. Thus when $[Ca^{2+}]$ was twice normal and $[K^+]$ was normal the proportion was practically the same as it was when $[Ca^{2+}]$ was 0·5 normal and $[K^+]$ was 0·25 normal. Again when $[Ca^{2+}]$ was normal and $[K^+]$ was normal the proportion fibrillating was about half as great as it was when $[Ca^{2+}]$ was 0·25 normal and $[K^+]$ was also 0·25 normal. The conflict between the effect of K^+ and that of Ca^{2+} is shown in the experiments in Table 1, in which the fibrillatory action of lowered $[K^+]$ was cancelled by lowering $[Ca^{2+}]$. However, the ratio between $[Ca^{2+}]$ and $[K^+]$ was found to have a limited importance, since when the ratio was the same as in the normal solution, the proportion of hearts fibrillating varied from 0 to 100% according to the absolute amounts of K^+ present, being 0 when $[K^+]$ was twice normal, and 100% when $[K^+]$ was 0·5 normal.

The interpretation of the observations on changes in Ca^{2+} which are summarized in Fig. 1 is clearly impossible at the present time. It is particularly difficult to understand the relations at Ca^{2+} concentrations less than 0·55 mM. For the proportion of hearts fibrillating appears to be greater the greater the concentration of K^+. Very probably two or even more factors are involved; one of these is likely to be membrane permeability, but the others are unknown.

In carrying out the experiments we received the impression that fibrillation occurred the more readily the more vigorous the ventricular contractions, and that a weakly beating heart did not fibrillate easily. The finding that fibrillation was arrested by removing Ca^{2+} from the solution, which was previously described (Armitage *et al.* 1957 *a*), may have been connected with the weakness of the beat in the absence of Ca^{2+}.

Previous observations (Armitage *et al.* 1957 *b*) have been confirmed that when stimulation did not cause fibrillation, the reduction of the $[Na^+]$ from 158 to 125 mM or lower resulted in stimulation causing fibrillation. A drop in Na^+ concentration had an effect in the same direction as a drop in K^+ concentration and was also counteracted by a drop in Ca^{2+} concentration. These results are shown in Table 2. It was therefore very surprising to read a paper by Grumbach (1957) on the defibrillatory action of Na^+ reduction. Grumbach produced fibrillation in most of his experiments by injection $CaCl_2$ into the cannula leading to the heart. He did not give figures showing how long such fibrillation lasted in different hearts. From experience in this laboratory with electrical stimulation, it would be expected that in many hearts fibrillation would last only for a short time and they would revert to normal rhythm. He described and illustrated one experiment in which he said the result was 'clear cut'. In this experiment he changed the perfusion fluid through a fibrillating heart to one containing 60·4 mM-Na^+, and then after 20 min observed a reversion to normal rhythm. Since reversion occurred only after 20 min it may have been a spontaneous reversion. Grumbach did not give figures for other hearts. During the present work experiments have been carried out in which fibrillating hearts have been perfused with a low (59 mM) Na^+ solution, and though in one or two experiments it was observed that the e.c.g. waves became less irregular and of larger amplitude, a cessation of fibrillation was seen in one experiment only; in this, however, fibrillation was re-established by stimulation during perfusing with the same low Na^+ solution, and it then persisted indefinitely.

Burn (1957) has suggested that the increased ease with which fibrillation is produced by stimulation when the heart is perfused with a solution low in Na^+ may perhaps be explained by the effect of the low Na^+ concentration on the active transport of K^+. If less Na^+ enters the cell, less Na^+ will need to be pumped out; since the amount of Na^+ pumped out may determine the amount of K^+ pumped in, as it has been shown to do in the nerve axons of *Sepia* (Hodgkin & Keynes, 1955), it follows that a low external concentration of Na^+ will lead to a lessened active transport of K^+. Now fibrillation in the atria has been shown to occur at a time when the net loss of K^+ exceeds a certain threshold (Holland, 1957) and fibrillation in the ventricles due to low $[Na^+]$ has been shown to be accompanied by an increased loss of K^+ (Armitage *et al.* 1957 *b*). Hence the facilitation of fibrillation by low Na^+ may be related to an increased net loss of K^+.

In addition to the foregoing results Mg^{2+} was observed to neutralize the fibrillatory action of Ca^{2+}. Before a further analysis of these observations can be made a study is required of the origin of fibrillation by means of microelectrodes. When this has been done, the effect of changes in the external ions on the membrane potential and on the critical level of excitation can be determined.

SUMMARY

1. When a rabbit heart is perfused, and the ventricles are stimulated electrically at a high rate, fibrillation occurs. The fibrillation either quickly reverts to normal rhythm or continues indefinitely according to the composition of the solution used for perfusion.

2. Fibrillation is encouraged to persist if there is a reduction in the concentration of K^+ or of Na^+ or of Cl^-. Fibrillation is also encouraged to persist if there is a rise in the concentration of Ca^{2+}. The effect of a rise in the concentration of Ca^{2+} can be neutralized by adding Mg^{2+} to the perfusion fluid.

3. In the presence of 2·2 mM-Ca^{2+}, the percentage of hearts which fibrillate persistently increases from 0 to 100 as the K^+ concentration falls from 11·2 to 1·4 mM. In the presence of 1·4 mM-K^+, the percentage of hearts which fibrillate persistently rises from 0 to 100 as the Ca^{2+} concentration rises from 0·1375 to 1·1 mM. In the presence of higher concentrations of K^+ the percentage of hearts which fibrillate is less dependent on the concentration of Ca^{2+}, and in the presence of normal K^+ (5·6 mM) the percentage of hearts which fibrillate rises very little as the Ca^{2+} concentration rises from 0·068 to 2·2 mM.

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A method for measuring arterial impedance using a differential manometer.

By D. H. Bergel, D. A. McDonald and M. G. Taylor. *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

By analogy with alternating current theory we can define the fluid impedance of any part of the arterial tree if we know the oscillatory flow and pressure so that for each harmonic term we write

$$\text{input impedance } (Z_0) = \text{pressure } (P)/\text{flow } (\bar{V}).$$

A study of the variation of impedance with frequency can give valuable information about the site and nature of reflexions in the arterial tree. In many arteries, however, it is difficult to measure flow directly, but it may be calculated from the pressure gradient using the method described by McDonald (1955) and Womersley (1955).

In the method demonstrated the pressure gradient is measured with a differential manometer over an interval of some 5–8 cm between the two recording points. At one of these the arterial pressure is also measured with a capacitance manometer.

The differential manometer used is an optical one in which the deflexion of the light beam is detected photoelectrically. This was originally described by Müller & Shillingford (1954), and is now commercially available from the Cambridge Scientific Instrument Co.

The pressures from the two recording sites are applied simultaneously to either side of a single metal membrane. The one we have used gives a full-range deflexion with ± 30 mm Hg. Static calibration is linear over this range. The natural frequency of the catheter-manometer system filled with fluid is about 50 c/s and careful calibration has shown that the response is flat to 10 c/s and almost certainly beyond this point. As we are only analysing results to the fifth harmonic of the pulse-frequency, i.e. 10–15 c/s, this is satisfactory. The instrument incorporates a variable (10-position) electrical filter. At the lowest setting this introduces a phase-error of about 0·5° per c/s. At higher settings this is much increased, e.g. for 8 c/s at position 5 the phase error is more than 60°, and the amplitude is reduced by 50%. It follows that in this type of work this filter cannot be used.

The arterial pressure and the differential pressure are displayed on a 2-beam cathode-ray oscilloscope and recorded photographically on film. Enlarged curves are drawn by projection and then analysed with an analogue computer (Taylor, 1958).

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Studies on primary hyperoxaluria.

By H. E. Archer, E. F. Scowen and R. W. E. Watts. *The Medical Unit, St Bartholomew's Hospital, London, E.C.* 1

Primary hyperoxaluria (Archer, Dormer, Scowen & Watts, 1957*a*) is characterized biochemically by the occurrence of a continuous high urinary oxalate excretion which probably dates from birth.

Variations in oxalate intake from the usual dietary sources do not provide a satisfactory explanation of the continuous high urinary oxalate excretion levels which are encountered in primary hyperoxaluria (Archer, Dormer, Scowen & Watts, 1957*b*). It has been shown that these patients do not absorb orally administered oxalate abnormally readily, and it is suggested on the basis of recent experimental evidence that glycine may be the precursor of at least some of the urinary oxalate in primary hyperoxaluria.

The present demonstration comprises summaries of the experiments which have been carried out in order to elucidate the aetiology of the disease, and the pedigrees of three families in which proven examples of the condition have arisen and in which the urinary oxalate excretion levels of relatives of the propositi have been measured. The latter data, although at present rather scanty, are compatible with primary hyperoxaluria being due to the operation of a rare recessive character. (See also Archer, Dormer, Scowen & Watts, 1958.)

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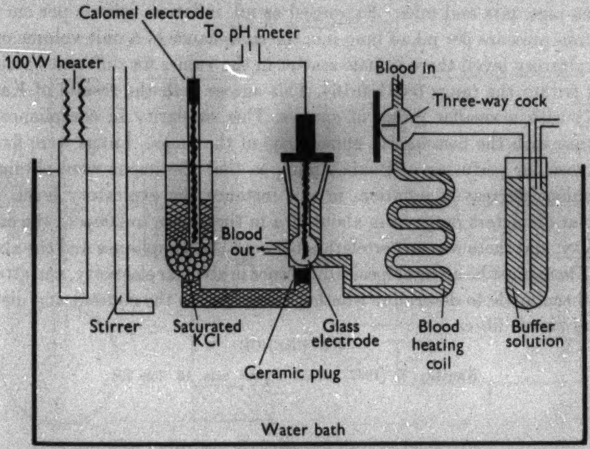
A method for the continuous determination of the pH of the blood.

By DAVID MENDEL. *The Medical Unit, St Bartholomew's Hospital, London, E.C.* 1

The electrode system and glass coil are built into a water-bath and the whole apparatus kept permanently at 37·5° C by means of an energy regulator and a 100 W heater and a stirrer.

Heparinized blood is fed, either by gravity or under arterial pressure, into the glass coil (90 cm long and 1 mm in internal diameter). During its passage through the coil the temperature of the blood reaches 37·5° C.


Calomel electrode
To pH meter
100 W heater
Blood in
Three-way cock
Blood out
Stirrer
Saturated KCl
Glass electrode
Blood heating coil
Buffer solution
Ceramic plug
Water bath

The electrode chamber is shaped like a small test tube with a ground glass joint at the top into which the glass electrode fits. There are three side arms on the chamber. To one of these the calomel electrode is connected via a ceramic plug and a tube of saturated potassium chloride. The blood enters through the second side arm and leaves through the third. The dead space of the coil and glass electrode system is about 3 ml.

A tube of buffer solution is immersed in the water-bath and the buffer can be introduced into the coil and electrode system at 37·5° C.

The rate of blood flow which is adjusted by means of a screw clip on the incoming blood tube determines the delay in the detection of any change in pH.

If the blood flows too quickly through the apparatus thermal equilibrium will not be reached and readings will be too high. The maximum permissible rate of flow will depend on the temperature of the blood and this rate is that at which no change of reading occurs when blood flow is stopped.

The accuracy of readings is ±0·02 pH unit.

The relationship between lung compliance and histology in different species.

By F. J. AUMONIER, M. L. CROSFILL and J. G. WIDDICOMBE. *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

The compliance of the lung has been measured in monkeys, dogs, cats, rabbits, guinea-pigs, rats and mice. Expressed as ml. inflation volume per cm water inflation pressure per ml. of lung (i.e. the compliance of a unit volume of lung at expiratory level) there is little scatter in the values for different species, all lying within the range 0·071–0·164. This agrees with the results of Kautsky (1957) with a smaller group of species. This similarity in compliance is in contrast with the histological appearance of the lungs. Lungs were fixed by intravascular perfusion of Bouin's fluid, so that specimens were obtained at equivalent degrees of inflation, in this instance the expiratory level. Mean alveolar diameters range from about 40 μ in the mouse to 200 μ in the cat and monkey, and there was no correlation between the compliance and the alveolar size. There must be an interspecies difference in alveolar elasticity, and attempts have been made to determine whether this is due to the content and distribution of elastic fibres.

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A method for recording changes in bronchial tone.

By J. G. WIDDICOMBE. *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

Most methods for recording alterations in bronchial tone in small animals measure changes in compliance (e.g. Konzett & Rössler, 1940). However, lung compliance is but slightly affected by bronchial tone (Radford & Lefcoe, 1955) unless the bronchi are constricted sufficiently to close off ventilatory units, and it is preferable to measure the resistance to airflow in the airways, since this is more directly related to the bronchial diameter and muscle tone.

The method demonstrated assumes (as a simplification) that the lungs consist of a resistance and a compliance (R_1 and C_1 in Fig. 1) in series. These are balanced by an 'artificial lung' consisting of a variable resistance (R_2) and compliance (C_2); the latter is a glass air-reservoir, in which the volume of air can be varied by adding or removing water. The animal's lungs and the artificial lungs are made part of a bridge circuit with two fixed and equal resistances (R) and the system subjected to constant pressure (10 cm H_2O) pulses of air; between every two pulses the system is opened to the atmosphere. Differential pressure is measured between the two sides of the circuit, and when the artificial lung is adjusted to match the mechanical properties of the real lung there will be minimal pressure oscillation. As soon as the airway resistance or compliance changes the circuit will be out of balance, and pressure swings will appear during inflation and deflation. If R_1 and R_2 are unequal there is a sudden short-lived (0·1–0·2 sec) spike of pressure, while if C_1 and C_2 are unequal there is a smaller and slower (0·5–1·5 sec) pressure change. These effects are as predicted by theoretical analysis of the system, and are obtained from the intact animal when airway resistance and lung compliance change respectively. They can be readily identified and distinguished. Since the circuit is originally balanced with minimal pressure swing the differential pressure recorder can be of high gain, so that small changes in airway resistance and compliance can be observed. The method can be made quantitative, subject to the oversimplification involved in the assumption that the lungs act as a resistance and a compliance in series.

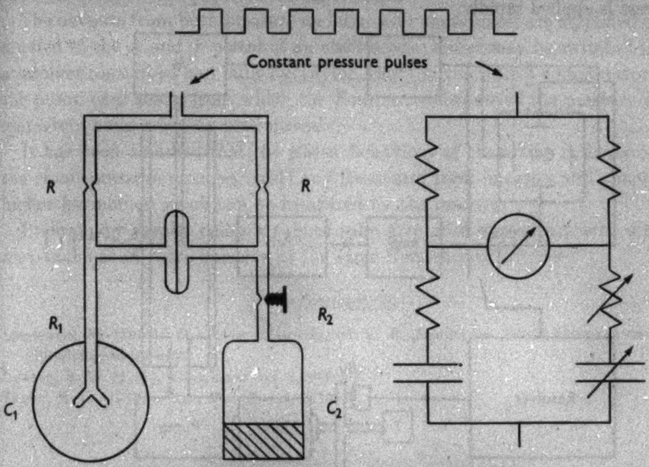

Fig. 1. Mechanical circuit with its electrical analogue.

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A photo-electric method for the determination of the elasto-viscous behaviour of the arterial wall. By D. H. Bergel.

Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C. 1

The elastic behaviour of the arterial wall is dependent on the time course of the applied stress, and the stress/strain curve obtained under dynamic conditions will form a hysteresis loop. This curve may be analysed using, in the first instance, the assumption that the arterial wall consists of elastic and viscous elements in parallel, viscosity permitting a smaller and delayed strain if the stress is applied rapidly.

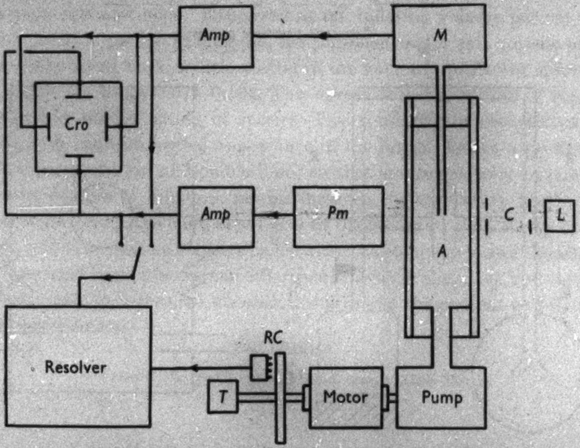

Fig. 1. *M*, manometer; *PM*, photomultiplier; *C*, collimating slits; *L*, light source; *A*, arterial segment; *RC*, resolver commutator; *T*, tachometer.

Thus the arterial wall is effectively stiffer at higher frequencies, and we may speak of a frequency-dependent modulus of elasticity which may be compared with Young's Modulus obtained under static conditions. The increase in wave velocity in an elasto-viscous tube with increase in frequency (Landowne, 1957) is partly attributable to this increased modulus.

Lawton (1955) has measured these effects using strips of aortic wall, but more directly physiological information may be obtained using whole vessels.

In the apparatus demonstrated, which is shown schematically in Fig. 1, a segment of vessel is extended to the length occupied in the body. A sinusoidally varying distending pressure, at frequencies throughout the physiological range, is applied and the diameter changes produced are measured, whence the stress-strain relation of the wall may be computed, using the equation: $T = PR$, where T = wall tension, P = distending pressure, R = radius, from which an expression which takes account of wall thickness is derived.

Arterial diameter is measured photo-electrically using a photomultiplier tube (R.C.A.-931-A) exposed to a diffusing screen on which the shadow of the artery is thrown by a shallow collimated light beam. The output of this unit is linear with vessel diameter and it is calibrated using standard rods and a resistance bridge.

The artery is distended by a sine-wave pump (Taylor, 1957) and the pressure measured with a capacitance manometer, using a needle whose tip lies within the vessel segment exposed to the light beam.

The outputs from both pressure and diameter transducers are amplified and applied to the X and Y plates of an oscilloscope; either may be switched into a resolver (described and built by Taylor, 1957) driven from a commutator on the pump cam shaft, from which the Fourier coefficients of the pressure and diameter changes can be read directly.

It has been assumed that the elastic behaviour of the artery is linear over the small pressure changes used; any departure from linearity will generate higher harmonics which can be measured by the resolver.

Preliminary results using a rubber tube give good agreement with direct measurement of wave velocity at the same frequencies.

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A simple electrical computer for Fourier analysis and synthesis.

By M. G. Taylor. *Department of Physiology, The Medical College of St Bartholomew's Hospital, London, E.C.* 1

This apparatus has been constructed for the rapid analysis and synthesis of wave forms in the study of the arterial circulation (McDonald & Taylor, 1957). Its greater advantage is the speed of operation; a 30-point analysis or synthesis, to five harmonics, can be performed in 15–20 min, as against 2–3 hr using an electric desk calculator. It is an enlarged and slightly modified version of the instrument described by Rymer & Butler (1944).

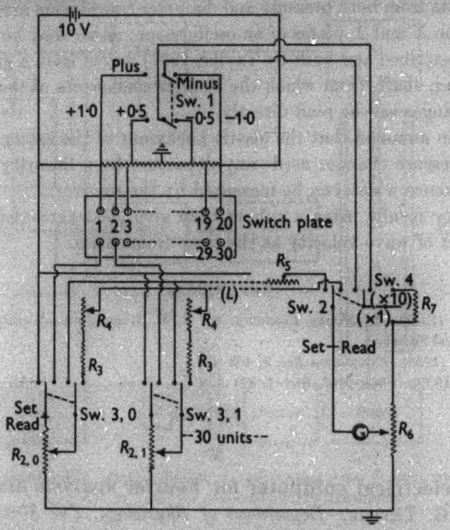
To find the first five pairs of Fourier coefficients, a_n, b_n of a wave form, given at thirty ordinates y_k equally spaced over a cycle,

$$y_k = \bar{y} + \sum_{n=1}^{15} \{a_n \cos(2n\pi k/30) + b_n \sin(2n\pi k/30)\} \quad (k = 0, 1, 2, \ldots, 29), \qquad (1)$$

we require the sums

$$a_n = \frac{1}{15}\sum_{k=0}^{29} y_k \cos(2n\pi k/30) \quad \text{and} \quad b_n = \frac{1}{15}\sum_{k=0}^{29} y_k \sin(2n\pi k/30).$$

That is, each of the thirty values y_k must be multiplied by a sine or cosine, and the sum averaged. In the circuit shown, the tapped resistor (R_1; 16 Ω) provides the 30 voltages corresponding to the sines and cosines of the angles ($2n\pi k/30$). These tappings are brought out to a switch, consisting of a Perspex plate with mercury-filled holes; connexions to the 30 potentiometers are made by a set of ten pre-wired contact-plates, one for each coefficient.



To set the instrument, Sw. 1 is put at 'Plus', Sw. 2 at 'Set', all Sw. 3 at 'Read' and Sw. 4 at 'times 1'. The values y_k, scaled to lie between 0 and 1, are applied in turn as fractional settings of the calibrated potentiometer (R_6; 10 KΩ), to 0·5% accuracy. With a value of y_k thus set, the corresponding potentiometer (R_2, k; 20 KΩ), with its switch (Sw. 2, k) at 'Set', is adjusted to give a null-reading of the galvanometer (G); its fractional setting then corresponds to y_k. This is repeated until all thirty have been set.

With Sw. 2 at 'Read', a contact-plate is inserted so that sine or cosine voltages are applied to the potentiometers in the order required by the series for the coefficient in question. The proportion of the sine or cosine voltage appearing at the slide of R_2, k represents the product of y_k (the fractional setting) and a sine or cosine. With all Sw. 2 at 'Read', the slides are connected through the adding resistors (R_3; 10 MΩ) to the common line (L). The voltage on this line is thus equivalent to $(1/30)\Sigma$; $(1/15)\Sigma$ is obtained by connecting R_6 to the 0·5-tapping of R_1. With appropriate polarity (using Sw. 1) the fractional setting of R_6 which balances the voltage on (L) now corresponds to a Fourier coefficient. If a reading on R_6 is less than 0·1, the scale may be multiplied by ten by adding (Sw. 4) the resistor R_7 (90 KΩ). Loading effect due to cascading of elements is very small with the values used for R_1, R_2, k and R_3. The adding resistors are accurately matched, using the trimming potentiometers R_4 (0·5 MΩ).

Fourier synthesis is performed by setting the ten coefficients on one or other of ten pairs of multiplying potentiometers, depending on the sign. These are connected together to the succession of 30 combinations of sine and cosine voltages required by the series (1), using a second, larger mercury switch. For each combination a balance of R_6 is obtained which represents an ordinate of the synthesized wave form.

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A simple method of time coding for use with continuous film records.

By A. J. Buller and P. Styles. *Sherrington School of Physiology and Medical Electronics Section, Department of Physical Medicine, St Thomas's Hospital, London, S.E.* 1

The apparatus has been developed in order to obtain photographic records of the intervals between successive impulses in a time series.

Fig. 1 shows a block diagram of the circuit used. Four Venner transistorized decade stages Type T.S. 10 are fed from a 10 kc multivibrator. The staircase output from the first decade scaler is displayed on the Y_2 beam of a Cossor 1049 double-beam oscillograph, and the resistively mixed staircase outputs from decade stages 2 and 4 are displayed on the Y_1 beam.

An impulse applied to the input flip-flop (Fig. 1, F.F. 1) produces a square-wave output which biases off the multivibrator. With no further incoming pulses from the multivibrator the scaler units hold their final reading until reset by a circuit (Fig. 1, F.F. 2) actuated by the trailing edge of the F.F. 1 square wave. At the end of the resetting pulse the decade units again count the output pulses of the multivibrator which is restarted at the end of the F.F. 1 square wave. This counting continues until a further impulse is applied to the input, when the cycle of operation is repeated. The duration of both the square wave and the resetting pulse may be varied, their respective values being 5 msec and 1 msec in Fig. 2.

Fig. 2 illustrates a typical record. Each vertical displacement of the small dots on the upper trace represents 1 msec, those on the lower trace, which can just be resolved, 0·1 msec. At *A* an incoming impulse turned off the multivibrator and a large dot on the upper trace indicates the holding time of

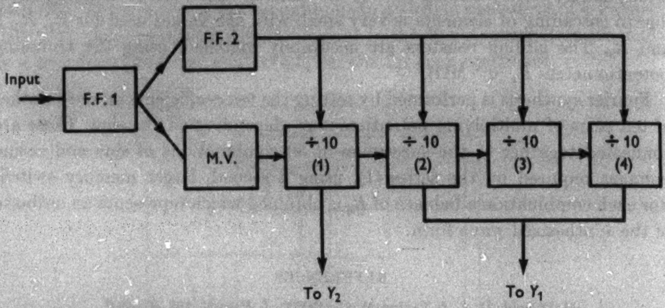

Fig. 1. Block diagram of circuit. F.F. 1 = input flip-flop; F.F. 2 = resetting circuit for scalers; *M.V.* = 100 kc multivibrator; (1) (2) (3) (4) = decade scaler units (Venner type T.S. 10) Y_1 and Y_2 = two beams of double-beam oscillograph.

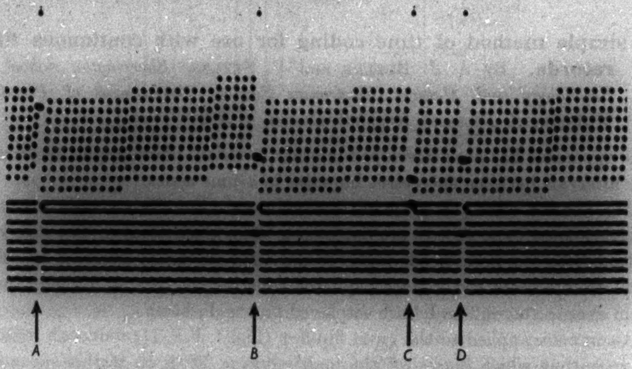

Fig. 2. Reproduction from 35 mm film of coded time display. For explanation see text. Interval *AB* = 258·7 msec, *BC* = 177·0 msec, *CD* = 60·7 msec. Film speed 2½ in. sec.

5 msec. After a resetting time of 1 msec, shown by the uppermost dot, the scalers recommenced to count and 252 msec passed before another incoming impulse again turned off the multivibrator at *B*. On the lower trace, counting from below upwards at *B* a reading of 0·7 msec is indicated. The total interval

between the two impulses was therefore 5+1+252+0·7 msec. Subsequent intervals illustrated are 177·0 and 60·7 msec.

By means of this apparatus possible errors in interval measurement due to variation in the film transport speed through the camera are eliminated, and the transport speed necessary for the timing accuracy obtainable is greatly reduced as compared with the more usual methods employed.

The use of a Gibbon type 'heart-lung' machine in experimental cardiac surgery.

By I. M. Hill, R. L. Hirt, O. S. Tubbs and B. G. Wells. *Department of Surgery, St Bartholomew's Hospital, London, E.C.* 1

A method of recording whole-body movement of mice in relation to ageing.

By Patricia J. Lindop and J. Rotblat. *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

Whole-body irradiation of animals by 15 MeV X-rays from the linear accelerator.

By Patricia J. Lindop and J. Rotblat. *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

The response of the male mouse breast to human mastotrophic substances.

By E. M. Donath, J. Hadfield and E. F. Scowen. *Departments of Medicine and Surgery, St Bartholomew's Hospital, London, E.C.* 1

A considerable variation in response between different strains of mice to a non-oestrogenic mastotrophic agent has been reported. This inability to react might be conditioned by other hormonal factors in the mouse, rather than some genetic property specific to a few reacting strains.

Fig. 1 shows the effect of progesterone in a dose of 1 mg per animal given by injection over 5 days on five breeds of non-reacting mice.

The next part of our experiment was designed to find out whether these non-reacting animals, when previously primed with progesterone, would react when stimulated by the human mastotrophic agent contained in a suspension in saline of desiccated human pituitary gland (Table 1).

Table 1. Effect of human anterior pituitary suspension on the breasts of S.A.S.4 mice

	Progesterone	Pituitary	Progesterone ↓ pituitary	Progesterone ↓ urine	Progesterone ↓ growth hormone
Club count	9	4	17	16	10
Degree of acinization	0	0	+++	++	++

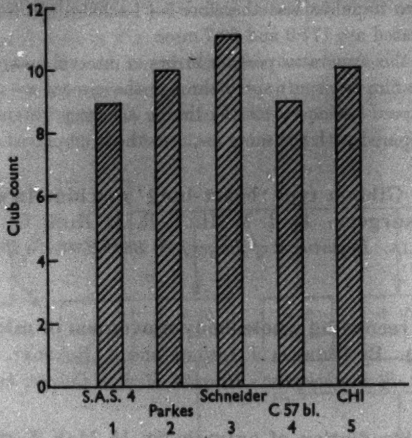

Fig. 1. Reaction in five breeds of mice to progesterone (1 mg/ml.). Control counts: for 1, 2, 4, 5 = 0; for 3 = 3.

A combination of human pituitary extract in an animal previously primed with progesterone produces an effect greater than the sum of the reactions of either agent singly. A similar effect was produced using growth hormone provided by Professor F. G. Young.

Variations, in the vertical work done against gravity and in the energy expenditure of walking, between men and women and in relation to training. By J. E. Cotes, F. Meade and M. E. Wise. *Pneumoconiosis Research Unit of the Medical Research Council, Llandough Hospital, Penarth, Glamorganshire*

Energy expenditure of horizontal walking (E_{O_2} cal/min), vertical lift per step (m) and work done against gravity (W_L = product of body weight, lift per step and step frequency, cal/min) were measured by Benedict & Murschhauser (1915); studies by the present authors (1957) suggest (*a*) that, given body weight and step frequency, W_L can be calculated, for a subject walking naturally, from a knowledge of leg, foot and pace lengths, and (*b*) that, at spontaneously chosen step frequency $E_{O_2} = aW_L + b$ cal/min (i) over the range 1–4 m.p.h. (1·6–6·4 km/hr).

During an investigation of training (Cotes, 1956) we found values for a and b for treadmill walking of 3·25 and 2·40. The subjects were ten volunteers aged 18–19 who had completed a 2-month training period. During this period three other sets of measurements were made, at 3½ m.p.h. (5·6 km/hr) only. The

W_L values at this velocity decreased significantly with time (mean values 0·91, 0·80, 0·73 and 0·65 cal/min, $P = 0{\cdot}01$), although the body weights of the subjects increased slightly.

The reduction was due to a decrease in lift work per step: the step frequency did not change significantly. The E_{O_2} values apparently declined ($P = 0{\cdot}11$, means 5·20, 5·01, 4·92 and 4·57 cal/min.) but in agreement with equation (i). Thus the values for a and b did not appear to change during training.

These findings support the suggestion (Knehr, Dill & Neufeld, 1942) that where a reduction in the energy expenditure of walking occurs during training it reflects a reduction in energy requirement because of better muscle co-ordination rather than increased muscular efficiency.

The suggestion of Booyens & Keatinge (1957) that, for anatomical reasons, the energy expenditure of women per kg body weight when walking at 3½–4 m.p.h. is less than that of men can also be substantiated. Lift work has been calculated using these authors' published data, with foot lengths (d) predicted from the empirical relationship $d = 0{\cdot}128$ height $+ 0{\cdot}04$ m ($r = 0{\cdot}70$). To allow for differences in body weights, the regression coefficients a of energy expenditure on lift work have been calculated using values for b estimated from the empirical relationship $b = 2{\cdot}39A - 1{\cdot}62$ cal/min ($r = 0{\cdot}80$). (A = body surface area, m^2).

Values for a for men and women walking at 3·4 and 4 m.p.h. are respectively 3·62 and 3·58, and 3·80 and 3·64. The similarity suggests that, at these walking speeds, energy expenditure per kg body weight for women is less than that for men because, at their natural step frequency, they perform less work.

We are indebted to the Army Personnel Research Committee for their co-operation.

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Changes in gastric function during pregnancy and lactation in women.

By J. N. Hunt and F. A. Murray. *Physiological Laboratory, Guy's Hospital, London, S.E.* 1

Effect of thyroid state on the contractility of isolated rat ventricle strips.

By J. M. Benforado. *Department of Pharmacology, Harvard Medical School*

Whitehorn & Ullrick (1955) noted that right ventricle strips taken from hyperthyroid rats developed less tension than controls. This report concerns similar experiments in which hypothyroid as well as hyperthyroid animals were employed. Further, tension development was studied over a wide range of frequencies (1–330/min) and at three different resting tensions ($\frac{1}{2}$, 1 and 2 g). The method used was that of Feigen (1952) with the exception that isometric tension was measured with a force-displacement transducer and ink-writing oscillographic system. Frequency of stimulation was controlled with a square wave stimulator. Changes in thyroid state were induced by the addition of 1% USP Thyroid powder or 0·1% *n*-propyl-thiouracil to the diet. Controls received no medication.

For all groups studied, when the resting tension was increased there was an increase in the tension developed. This effect was most marked at lower frequencies. The main effect of increasing the frequency of stimulation was a decrease in developed tension. There was, however, a maximum tension which in the control groups was at frequencies of 7–17/min. Below these frequencies the tension was less. The hyperthyroid groups showed this maximum at 35–70/min, while for the hypothyroid groups, this was at 1/min, the lowest frequency studied.

Comparison of the mean tensions developed indicated that, at the same resting tension and frequency, the hyperthyroid groups developed less tension than controls. In one series of experiments, these differences were significant at $P<0{\cdot}01$ over the entire frequency range employed. A similar comparison for the hypothyroid groups showed a varied picture. In one series, a consistent though not significant increase in tension over controls was noted. In another series, increases ($P<0{\cdot}10$) were observed only at lower frequencies.

These differences in tension development of isolated rat ventricle strips taken from animals in varying thyroid states may be related to the uncoupling of oxidation from phosphorylation by the thyroid hormone (Brody, 1956).

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The separate release of oxytocin and ADH. By G. W. THEOBALD. *St Luke's Maternity Hospital, Bradford.*

Notwithstanding the synthesis of oxytocin and the determination of the formulae for ADH it is still not decided whether these two substances can be liberated separately. Abrahams & Pickford (1954), working with dogs, found that appropriate osmotic stimnli invariably caused the release of 'from 15 to 20 times as much oxytocic as antidiuretic material'.

We have found that the intravenous delivery of 5 m-u. a minute of oxytocin (either Pitocin (Parke, Davis) or the synthetic Syntocinon (Sandoz Products) usually suffices either to induce labour or to enhance the effectiveness of uterine contractions. The fact that the oxytocin drip was employed argued the existence of some degree of abnormality in the patient.

The following investigations were carried out in an attempt at assessing the physiological amount of oxytocin necessary to initiate labour and effect delivery. The oxytocin drip was instituted: (*a*) in patients who had had their membranes artificially ruptured; (*b*) after the pains of early labour had been temporarily inhibited by the injection of a local anaesthetic; (*c*) in patients with secondary uterine inertia; and (*d*) during the early days of the puerperium. In the last group uterine contractions were recorded by means of a balloon in the uterus, and simultaneous water diuresis curves were obtained. The effect of suckling on these two activities was matched by the intravenous administration of oxytocin and ADH. It was concluded that the human myometrium at term normally becomes sensitive to, and the let down of milk is caused by, the intravenous injection of from 0·2 to 0·1 m-u. each minute of a commercial preparation of oxytocin. The amount of ADH which causes minimal inhibition of water diuresis is of a strikingly similar order. It is nevertheless believed that the separate release of oxytocin and ADH does occur, for suckling causes either no antidiuresis or significantly less than would be expected, while on the other hand antidiuresis provoked by the rapid intravenous injection of a litre of 2·5 % NaCl caused no uterine contractions.

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Cystathionine in brain and liver from pyridoxine-deficient rats.

By D. B. Hope. *Department of Pharmacology, University of Oxford, and M.R.C. Radiobiological Research Unit, A.E.R.E., Harwell, Didcot, Berkshire*

Although generally believed to be an important intermediate in trans-sulphuration, L-cystathionine was only recently isolated from mammalian urine in pyridoxine deficiency (Blaschko & Hope, 1956; Hope, 1957*a*). It has since been found in relatively high concentration in the brains of man and rhesus monkey (Tallan, Moore & Stein, 1958).

The livers and brains from pyridoxine-deficient rats have now been examined. Cystathionine was found in both organs.

Cystathionine was demonstrated by two-dimensional paper chromatography, using phenol saturated with water, followed by *n*-butanol–acetic acid–water (4:1:5 by volume; upper phase). In the presence of added cystathionine no additional spot appeared in the chromatograms. Confirmation has been obtained by treatment of extracts with H_2O_2 (Dent, 1947); the cystathionine spot disappeared with formation of a new ninhydrin-positive substance with lower R_F values in either solvent (Fischer, 1957).

In the brain extracts the spot due to cystathionine was one of the most conspicuous spots on the chromatograms; there were no other ninhydrin-positive substances present with the same R_F in the second solvent. Advantage was taken of this observation for a quantitative estimation, similar in method to that previously described for taurine (Hope, 1957*b*). The brains of two female rats contained 64 and 86 mg, those of two male rats 61 and 65 mg, of cystathionine per 100 g wet tissue. Enough brain tissue was obtained from fifteen animals to isolate 3 mg of the amino acid. Infra-red spectroscopy confirmed the identity of the substance with cystathionine.

The cystathionine spot in the chromatograms of liver extracts was weaker, and the presence of other ninhydrin-positive substances with similar R_F, so far not identified, made a quantitative estimation impossible.

From these observations we must conclude that trans-sulphuration occurs at a high rate in brain tissue, as already suggested by the work of Gaitonde & Richter (1957). In the brain of man and monkey cystathionine normally appears to accumulate; in the rat this only happens when the ability to cleave the amino acid is impaired by the depletion of pyridoxal phosphate.

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The effects of stimulation of the carotid body chemoreceptors on heart rate in the dog. By M. DE BURGH DALY and MARY J. SCOTT. *Department of Physiology, University College London*

Although it is well known that anoxic anoxia causes tachycardia, there is no general agreement as to the mechanism of this effect. It has been attributed by some to stimulation of chemoreceptors (see review by Neil, 1956*a*) in spite of the original observation of Heymans, Bouckaert & Dautrebande (1931) that stimulation of the carotid body by various drugs injected into the common carotid artery caused bradycardia. More recently, however, Bernthal, Greene & Revzin (1951) and Neil (1956*b*) have concluded that anoxic tachycardia is not of chemoreceptor origin.

The present experiments were performed on dogs anaesthetized with a mixture of chloralose and urethane preceded by morphine hydrochloride. The carotid chemoreceptors were stimulated by changing the blood perfusing them from arterial to anoxic. For this purpose both carotid bifurcation regions were isolated from the circulation and perfused by a Bayliss-Müller roller pump. Connexions were made in such a way that either arterial blood from the animal breathing room air or anoxic blood from a femoral artery of a donor dog breathing 5 or 7 % O_2 in N_2 could be passed through the chemoreceptors.

It was found that, in animals with open or closed chest artifically ventilated by means of a Starling 'Ideal' pump, stimulation of the carotid bodies by anoxic blood invariably causes a profound bradycardia, which occurs independently of changes in systemic blood pressure. This response is reduced or abolished by atropine or by division of the cervical vagosympathetic nerves. It is also abolished by division of the carotid sinus nerves.

In dogs breathing spontaneously chemoreceptor stimulation causes either an increase, a decrease or no change in heart rate. There is also an increase in respiratory minute volume which is due in part to an increase in tidal air volume. Observations made during the course of these experiments suggested that the cardiac accelerator response was due, at least in part, to a stretch reflex from the lungs resulting from the increased depth of breathing (Anrep, Pascual & Rössler, 1936). Further experiments designed to test this hypothesis showed that the tachycardia occurring in response to carotid body stimulation was converted to a bradycardia after division of the pulmonary branches of the vagus nerves although little or no change occurred in the effect on respiration. In those experiments in which the control response to chemoreceptor stimulation was a reduction of heart rate, lung denervation enhanced the response.

These results indicate that chemoreceptor stimulation causes a primary reflex slowing of the heart and that this effect may be partly or wholly masked by a secondary reflex tachycardia originating in the lungs.

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Gustatory sweating following parotidectomy.

By D. H. Glaister, J. R. Hearnshaw, P. F. Heffron and A. W. Peck. *Department of Physiology, Middlesex Hospital Medical School, London, W.* 1

The motor supply to the extra-ocular muscle spindles.

By D. Whitteridge. *Department of Physiology, University of Edinburgh*

Changes in the distensibility of the uterine cervix of the rat after parturition.

By Margaret L. R. Harkness and R. D. Harkness.
Department of Physiology, University College London

Observations on the physical properties of the uterine cervix of the rat during pregnancy have been reported previously (Harkness & Harkness, 1955). It seemed of interest to extend investigation to the period of involution after parturition, when rapid change in the collagen content of the cervix is taking place (Harkness & Harkness, 1954; Harkness & Moralee, 1956).

The method was similar to that used before. The excised cervix was suspended in Locke's solution and pulled out between two parallel steel rods one through each canal. The tissue was first loaded with 50 g for 25 min and subsequently the load was increased by 25 g every ¼ min until the tissue ruptured. The inner circumference of the cervix (twice distance between centres of rods plus circumference of one rod) was taken as a measure of the length of the tissue. Most of the experiments were done at 22° C.

Cervices from groups of rats killed during parturition, and 2–4 hr, 6–8 hr, 1, 2, 3, 4, 8 and 16 days afterwards, were examined. Except during the first half of the first day after parturition the initial loading produced a change in circumference which was linear when plotted against the logarithm of time. Length–tension curves for the subsequent loading were nearly linear in all groups of rats. The curves from different groups of rats differed principally in their mean height. The circumference of the cervix dropped rapidly in the first 8 hr after parturition to about one third its initial value but subsequently changed more slowly, taking till the 16th day to fall by a similar proportion to about its normal value. Samples from littering and 8 hr post-partum rats stretched at 37° C and stretched in the presence of papaverine sulphate (50 mg/l.) showed a similar difference in circumference to those tested at 22° C. The changes in the first 24 hr were accompanied by increase in the concentration of collagen, which changed little thereafter. Breaking tension calculated per unit cross-sectional area of collagen was in general approximately constant though the samples from rats at 8 hr, and during parturition tested at 37° C, were relatively weaker.

The nature of the above changes is such that it appears that they must principally concern the connective tissue framework, which diminishes rapidly in size after parturition and within 24 hr forms new linkages which resemble in their properties those normally present. The reduction in size of the connective tissue framework takes place much more rapidly than the loss of collagen after parturition.

We are grateful to the Nuffield Foundation and the Medical Research Council for a grant for this work.

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The renal response of conscious dogs to occlusion of the common carotid arteries. By W. J. O'Connor. *Department of Physiology, School of Medicine, University of Leeds*

When 0·9% sodium chloride is administered to conscious dogs increased excretion of sodium always results, but the picture presented by the urinary changes depends on the state of salt-loading and the existing rate of excretion of sodium (O'Connor, 1958). Earlier, it was found that sodium excretion is increased by occlusion of both carotid arteries (O'Connor, 1955). It has now been found that the renal response to carotid occlusion at any state of salt-loading resembles the response to the ingestion of 0·9% sodium chloride: if the existing rate of sodium excretion is low (less than 0·02 m-equiv/min) both ingestion of saline and carotid occlusion produce increased excretion of sodium due to increased urinary sodium concentration without great increase in the volume of the urine; if sodium excretion is greater than 0·02 m-equiv/min, increased excretion of sodium is due to increased volume of the urine, without great change in the urinary concentration. Carotid occlusion always causes an increase in the excretion of potassium, but only a doubtful increase in the excretion of urea. Carotid occlusion increases the rate of urine flow in water diuresis.

Under all conditions the renal effect of carotid occlusion is about equal to the effect of the ingestion of 60 ml. 0·9% sodium chloride. Carotid occlusion produces an increase of about 40 mm Hg in arterial blood pressure and the ingestion of 60 ml. of saline produces a fall of about 0·15 g/100 g in the solid content of the plasma; it is suggested that the equal effects on urinary flow and composition may be best ascribed to equal effects of these two factors on glomerular filtration.

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Adaptation in man to exhausting physical work. By J. G. Fletcher. *Division of Human Physiology, National Institute for Medical Research, London, N.W.* 3

Three young men performed hard physical work by stepping on and off a bench 22 in. (56 cm) high, thirty times every minute. The subjects continued stepping until they were exhausted and tests were repeated daily. As the leading leg was the right, less physical work was performed by the left leg.

On the first occasion, the subjects succeeded in stepping for 2 min 10 sec, 1 min 50 sec and 2 min 40 sec. Endurance increased after a few days and 15 min stepping was achieved in the 33rd, 17th and 22nd experiment respectively. However, when the subjects then attempted to step with the left leg leading, they were able to continue only for 4 min 10 sec, 5 min 3 sec and 4 min 3 sec.

One subject, after an interval of 13 weeks, resumed step tests, and his initial time was now 7 min 3 sec. At the 12th experiment his stepping time was 13 min 17 sec. There was a second interval of 32 weeks, before further tests were begun, and his initial stepping time was now 5 min 19 sec. At the 12th test he was able to step for 25 min 49 sec.

Pulse rates were counted before each test and then between 1 and 1½ min after the conclusion of the test, in both cases in the sitting position. The initial pulse rate in the three subjects decreased by 4–10 beats/min from the beginning to the end of the first series of tests.

After the first series had been completed, the three subjects repeated their initial stepping times, and also carried out a 5 min test. In both cases there was in all three subjects a marked decline in the post-work pulse rate on the second occasion.

These results indicate that adaptation to exhausting physical work can be achieved by short daily periods of exercise, and that the adaptation persists for a long time.

The accuracy of the integrating motor pneumotachograph (Imp).

By H. S. Wolff. *Division of Human Physiology, National Institute for Medical Research, London, N.W. 3*

The integrating motor pneumotachograph or Imp (Wolff, 1955) is calibrated by blowing an intermittent stream of air through a dry gas-meter and the Imp in series, at various flow rates. The calibration factor for any one instrument is constant to within 1% for approximately a 12-fold range of air flow, i.e. 6½–80 l./min.

Two standard models of the Imp were adapted so that the air issuing from the outlet could be collected in a 500 l. Douglas bag. The subject wore the Imp and his ventilation volume was measured during sitting and walking or running on a treadmill. The duration of each experiment was determined by the ventilation volume, as approximately 425 l. was collected each time. The volume registered by the Imp was compared with the volume of gas in the Douglas bag, and the composition of the gas sample collected by the Imp (0·03% of total volume) was compared with the composition of a sample of the gas in the Douglas bag.

There were fourteen determinations of ventilation volume, in the range of 6·4–81·0 l./min, nine with one instrument and five with the second. The volumes measured in the Douglas bag were on the average 0·7 l. lower than those recorded by the Imp. When the individual differences were expressed as a percentage of the volume in the Douglas bag, the range of deviation for one instrument was from $-0{\cdot}2\%$ to $+0{\cdot}56\%$, and for the other from $-0{\cdot}5\%$ to $+0{\cdot}9\%$.

The composition of the gas samples obtained from the Imp and the Douglas bag was similar. The range of difference in percentage composition of the paired samples was from $-0{\cdot}04\%$ to $+0{\cdot}01\%$ for both O_2 and CO_2, when the Douglas bag values were subtracted from the Imp values.

The differences in the values of the ventilation volume and of the composition of the gas samples as measured by the Imp and Douglas bag method are very close to the experimental errors of measurement.

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The responses of the vascular system in different phases of the reproductive cycle. By Sybil M. Lloyd*. *Department of Physiology, University of Edinburgh*

This problem was approached by observing the effects of known vasoactive substances such as vasopressin and oxytocin. All observations were made on anaesthetized rats in which carotid blood pressure was recorded, and the mesenteric vessels observed directly. The vasopressin used was the highly purified natural substance kindly given by Dr V. du Vigneaud, and the oxytocin was the synthetic brand, Syntocinon, generously supplied by Sandoz Ltd.

In female rats throughout the reproductive cycle intravenous vasopressin produced a rise in blood pressure, with constriction of the mesenteric arterioles and capillaries. In dioestrus rats oxytocin had no effect on the blood pressure, but caused dilatation of the mesenteric arterioles; in oestrus rats it caused a pressor response, with arteriolar constriction.

For some days after ovariectomy the sensitivity to oxytocin was increased by a factor of approximately 5; at the same time the sensitivity to vasopressin was reduced, also by a factor of approximately 5. After a week, the sensitivity to both substances returned to normal.

In normal rats, subcutaneous injections of stilboestrol and progesterone, either singly or together, on many occasions caused the appearance of a pressor response to oxytocin. Where this occurred the sensitivity to vasopressin was increased. In ovariectomized rats stilboestrol and progesterone injected together, but never singly, converted the action of oxytocin to a pressor one.

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These results point to the importance of noting the phase of the reproductive cycle when testing the response of the vascular system to drugs and hormones.

The vascular action of 5-hydroxytryptamine on the canine liver.

By W. H. H. Andrews and K. R. Butterworth. *Pharmacology Department, St Mary's Hospital Medical School, Paddington, W. 2*

Many authors have stated that isolated perfused canine livers may rapidly develop congestion. This congestion is apparently due to constriction of the hepatic venous tree (Bauer, Dale, Poulsson & Richards, 1932; Andrews, Hecker, Maegraith & Ritchie, 1955) and develops especially rapidly when whipped blood is used for the perfusing fluid. Both histamine and 5-hydroxytryptamine (5-HT) are liberated by whipping, and accordingly the action of both was investigated on the canine liver. Histamine does give rise to congestion, but its action is both prevented and reversed by mepyramine maleate which has no action on the congestion due to the unknown agent.

The canine liver is considerably less sensitive to 5-HT than to many other substances, such as adrenaline. An injection of 15 μg gave rise to small effects, and 1 mg could be added to 500 ml. of perfusing fluid (blood or Ringer–Locke solution) without producing congestion. Recorded responses were: a decrease of liver volume, of both portal venous and hepatic arterial inflow and of hepatic venous outflow; an increase of inflow pressures.

The following experiment was performed on isolated livers. When the liver was perfused via the hepatic artery only and with a manometer introduced into the portal vein, the hepatic arterial pressure, the hepatic venous outflow and the 'static' portal pressure were related, and an increase of arterial pressure led to an increase of both static portal pressure and outflow. Introduction of 5-HT to the perfusing fluid caused a decrease in both 'static' portal pressure and outflow for any given arterial pressure, but the relationship between static portal pressure and outflow was virtually unchanged. When the liver was perfused via the portal vein, there being no arterial flow, an increase of portal pressure—now dynamic—gave an increase in outflow. Addition of 5-HT to the perfusion system decreased the outflow for any given dynamic portal pressure. From the portally perfused liver it can be deduced that 5-HT increases resistance to flow through the liver, whilst from the arterially perfused liver one may see that the increase in resistance probably does not occur after the artery and portal vein have joined, and therefore is not present in the hepatic vein.

The cause of hepatic congestion in perfused preparations is unlikely to be either histamine or 5-hydroxytryptamine. The latter drug produces constriction of the hepatic arterial and portal venous systems.

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The effect of 2:4-dinitrophenol and of potassium on the uptake of 5-hydroxytryptamine by platelets.

By G. V. R. Born and R. E. Gillson. *Nuffield Institute for Medical Research, University of Oxford*

The uptake of 5-hydroxytryptamine (5-HT) by human blood platelets was observed by methods already described (Born & Gillson, 1957). 5-HT was added to the fluid in which the platelets were suspended; the concentration of 5-HT was 1·5 μg/ml. When the fluid contained 1×10^{-5} M 2:4-dinitrophenol the platelets took up more 5-HT than when dinitrophenol was absent. The increase was in the initial rate of uptake rather than in the total amount taken up. After 90 min the average uptake was increased by 35% (range 10–63%). When the concentration of dinitrophenol was 1×10^{-4} M the rate of uptake was sometimes increased and sometimes decreased, and with 1×10^{-3} M dinitrophenol it was always decreased.

When the concentration of potassium in the suspending fluid was increased above 5·9 mM, which is the concentration of K in Krebs's bicarbonate solution, the rate of uptake of 5-HT by platelets was also increased. After 90 min the increase was greatest, in one experiment, in 15 mM-K (80% greater than in 5·9 mM-K); in another experiment, in 29·5 mM-K (80% greater); and in a third, in 59 mM-K (330% greater).

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The variation of radiation sensitivity with age.

By Patricia J. Lindop and J. Rotblat. *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

It is believed that the effect of whole-body exposure to ionizing radiations is to cause generalized tissue damage, some of which is irreparable. It is the accumulation of this permanent injury which is thought to be responsible for the shortening of life span due to radiation, as well as for the acceleration of the ageing process which occurs. According to this theory one would expect that the resistance to radiations, as, for example, expressed by the dose necessary to produce a given effect, should decrease with age. In order to verify this, mice of the same strain were given a range of similar doses of radiation at different ages. For each age group the LD 50 at 30 days after exposure was obtained. The results of this experiment will be presented and their interpretation, in the light of the present theories of ageing, discussed.

The expenses of this research were defrayed from a grant made by the Trustees of the Nuffield Foundation.

Circulatory, metabolic and respiratory effects of *iso*propylnoradrenaline in man.

By A. F. Cobbold, H. J. B. Galbraith, Jean Ginsburg and A. Paton. *Sherrington School of Physiology and Department of Medicine, St Thomas's Hospital, London, S.E.* 1

Intravenous infusions of (±) *iso*propylnoradrenaline (Isuprel, Winthrop) were given to healthy adults in a dose of 0·1 μg/kg/min for 30 min periods. Pulse rate and pulse pressure were almost doubled within 2 min of beginning the infusion and remained elevated throughout. Blood flow in forearm and in calf showed an initial marked increase, succeeded by a smaller but sustained increase at 300 and 100 % above the resting level respectively. Hand and foot blood flow increased initially but transiently; the sustained phase, seen in muscle blood flow, was absent. The peripheral vasodilatation may be partly attributed to the direct action of *iso*propylnoradrenaline on the blood vessels of skeletal muscle since forearm blood flow increased with intra-arterial infusions of doses comparable to those reaching forearm during intravenous infusion whilst hand blood flow was unaffected.

Oxygen consumption rose abruptly to about 66 % above the control level during the second minute of infusion and then subsided to remain at about 33 % above control. A slow rise in blood sugar occurred during intravenous infusion reaching 30 % above fasting sugar level; there was no change in blood lactate concentration.

Respiration was increased, mainly owing to an increase in depth. The increase began shortly after the start of the infusion and was maintained throughout.

Anaesthesia and local tetanus intoxication.

By B. L. Andrew and Margaret N. Barr. *Departments of Physiology and Anaesthetics, Queen's College, Dundee*

It is well known from electromyographic studies on experimental animals that the spontaneous continuous activity in the muscle affected by local tetanus intoxication is abolished by general anaesthesia. However, the nervous control of such a muscle in the anaesthetized animal is abnormal, since there is a level of anaesthesia at which stretch reflexes are absent in other muscles, yet are still present in the intoxicated muscle. This heightened excitability of the α-motoneurones serving the muscle might be due entirely to the direct action of the poison on the α-motoneurones (Brooks, Curtis & Eccles, 1957, have shown the isolation of the motoneurones from inhibitory pathways in the poisoned animal) or might in part be due to increased activity in the small motor fibres serving the muscle spindles in the affected muscle, and hence reflexly increasing the excitability of the α-motoneurones. To test this possibility three types of experiment were performed. Adult rats were used; local tetanus intoxication was produced in the flexor or extensor groups of muscles of one hind limb by the injection of tetanus toxin into the gastrocnemius or quadriceps muscles three days before the experiment. Urethane and pentobarbitone sodium, given intraperitoneally, were used to obtain light anaesthesia, trichloroethylene vapour was used to obtain transient deepening of anaesthesia.

In one group of rats preparations were made from the central ends of motor nerve branches to the quadriceps muscles in poisoned and normal limbs and comparisons made of the activity in small and large motor fibres.

In the second group the lumbar spinal cord was exposed and recordings were made from filaments dissected from the ventral roots serving the hind limb. Comparisons were made of the efferent activity to normal and poisoned sides.

In the third group afferent fibres from normal and poisoned limbs were dissected out from the dorsal roots. Attempts were made to distinguish muscle spindle afferents from continuously discharging fibres serving other modalities of sensation by their reflex response to stimulation of the γ-motor system through ear and skin.

These experiments, taken as a whole, did not reveal any obvious difference in the level of γ-fibre activity to poisoned and normal muscles. This suggests that the inflow of nerve impulses from the muscle spindles, while not greater in volume, is more effective in the poisoned region of the spinal cord, producing spasticity in the unanaesthetized animal or retaining sensitivity to stretch in the lightly anaesthetized animal.

We thank Miss M. Barr of the Wellcome Research Laboratories for the gift of toxin, and M. N. B. acknowledges a grant from the Scottish Hospital Endowment Research Trust.

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A preliminary investigation of the effect of isoprenaline on the breathing of chloralosed cats.

By J. DIMSDALE and MARY F. LOCKETT. *Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W. 3*

The spontaneous breathing of cats anaesthetized with 6·0–8·0 ml. 1% chloralose in 0·9% NaCl/kg was recorded by Gaddum's method. In these animals, the intravenous injection of 0·1–1·0 μg ±isoprenaline/kg caused an increase in the depth and rate of breathing which exceeded that caused by the same weight of the amine introduced into a carotid artery through a retrograde cannula in a lingual artery. The latent period of the threshold effect of isoprenaline on tidal volume was shortest when the amine was given intravenously. The effect of the amine on respiration was not reduced by section of moderator nerves, or by removal of the stellate ganglia, or by the intravenous injection of hexamethonium in weight sufficient either greatly to reduce or to abolish the submaximal effects of nicotine both on breathing and on mean arterial pressure. Neither section of the cervical vagi nor division of the spinal cord at the lower border of the sixth dorsal vertebra abolished the effect of isoprenaline on tidal air. The major part of the respiratory response to isoprenaline, as to adrenaline, has been shown to involve the second and third dorsal segments of the cord. Both adrenaline and isoprenaline produce their effects on breathing reflexly from receptors in the chest. Weight for weight (±) isoprenaline has always proved more potent than (−) adrenaline in this respect.

Factors affecting histamine-stimulated gastric secretion in anaesthetized dogs.

By J. W. BLACK, E. W. FISHER and A. N. SMITH. *Departments of Surgery and Veterinary Physiology, University of Glasgow*

A constant intravenous infusion of histamine was used to stimulate gastric acid secretion in dogs with vagi intact, anaesthetized with chloralose and urethane, and prepared with a simple stomach fistula and a ligature at the pyloro-duodenal junction. When the animals had starved for 24–36 hr before operation, the histamine-stimulated secretion reached a steady state between 1½–3½ hr after starting the histamine infusion; when the animals were not starved before the experiment, the acid output began to decline after 1–1½ hr and had ceased by 3–3½ hr after starting the histamine infusion (Table 1). This decline and cessation of histamine-stimulated secretion in recently fed dogs could be prevented by bilateral cervical vagotomy and by exclusion of acid from the pyloric antrum.

The usual dietary regime of dogs used for studies of gastric secretion is to give a single meal of meat per day and most studies on gastric secretion in dogs have been done on animals starved for periods between 18 and 36 hr. In our experiments the dogs which showed the lessened secretory response were fed twice per day with meat and milk and were not without food for more than 12 hr.

TABLE 1. Histamine-stimulated acid gastric secretion (*a*) from 'starved' dogs and (*b*) from 'fed' dogs. The figures are averages of the total output of free acid in m-equiv/15 min from two consecutive 15 min collection periods. The ½ hr time intervals refer to the start of the histamine infusion as zero

		Histamine 5 μg/kg/min intravenously							
Expt.	Dog wt. (kg)	½	1	1½	2	2½	3	3½	4
(*a*) 1	15	0·03	0·42	0·95	1·11	1·20	1·28	1·16	—
2	24	0·92	3·84	4·49	4·46	4·48	—	—	—
3	11	0·04	1·09	5·01	5·45	6·08	5·10	—	—
4	15	0·39	2·34	3·03	2·46	2·64	2·75	2·76	2·75
(*b*) 5	21	0·85	2·68	1·53	0·92	0·43	0·00	0·00	—
6	20	0·02	0·34	1·10	0·78	0·19	0·00	—	—
7	15	0·04	2·00	1·78	0·06	0·10	0·01	0·00	—
8	14	0·01	0·05	1·11	0·31	0·17	0·00	—	—

Inhibition of histamine-stimulated gastric secretion takes place in dogs anaesthetized with chloralose and urethane provided that there is no prolonged period of starvation before the experiment, that the vagi were intact, and that the continuity of the body and antrum of the stomach was not disturbed. There may possibly be a vagal pathway for the inhibitory effects following feeding. Code & Watkinson (1955) and Schachter (1949), have carried out experiments related to this problem. Schachter considered that the vagus contains secretory-inhibitory fibres in its supply to the stomach, as described by Pavlov (1902).

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Polyuria during experimental modifications of breathing. By

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In earlier experiments we found that hyperpnoea led to polyuria (Currie & Ullmann, 1955), and later, that this was independent of changes in blood gas tensions and not quantitatively related to changes in respiratory rate, depth or minute volume.

We have now investigated the effect of changes in inspiratory airway resistance on water excretion. Subjects sat upright, and drank 500 ml. of water at the end of an initial control period. Immediately following this the test procedure was begun and continued for one hour.

In control runs when breathing was normal throughout, the fraction of the water load eliminated 90 min after ingestion averaged 47%. It averaged: 88% when 10% O_2 in N_2 was breathed, with alveolar pCO_2 constant; 101% during voluntary overventilation, with alveolar pCO_2 constant; 87% when 4–6% CO_2 was breathed; 90% when inspiratory resistance was raised high by insertion of a glass capillary into the external airway. It averaged 78% when the same subjects breathed air at normal rate and depth through the valve used in these experiments.

Breathing through the valve alone without other procedure increased oesophageal pressure variations during the respiratory cycle by *ca.* 10 mm H_2O. The change was approximately 25% of the pressure swing without the valve.

It therefore appeared that a small increase in intrathoracic pressure fluctuation somehow induced polyuria, whereas much larger pressure changes had comparatively little additional effect.

In order to see whether greater responses failed to develop with larger pressure changes because the diuresis was self-limiting, further experiments were begun in which a water load of 7 ml./kg was maintained by drinking every 30 min as much fluid as was excreted. Test procedures started 2 hr after water loading and introduction of an oesophageal balloon and continued for 2 hr.

Experiments completed so far showed that increased diuresis could be maintained for 2 hr if the water load was kept up. It was confirmed (*a*) that breathing through the valve was associated with small oesophageal pressure changes, and led to increased water excretion; (*b*) that procedures associated with larger oesophageal pressure changes had but a small additional effect; (*c*) that urinary pH, creatinine, Na, K, and total solute excretion remained unaffected. Negative pressure breathing (−10 to −15 cm H_2O) was no more effective than breathing through the valve alone. Recumbency augmented diuresis to about the same extent as did valve breathing.

These results are compatible with the hypothesis that polyuria during hyperpnoea is mediated reflexly, in the manner suggested by Sieker, Gauer & Henry (1954) and Henry & Pearce (1956).

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Variation in the response of human tissues to similar stimuli at different ages.

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Biopsy specimens were taken from the gums of a series of patients attending the Dental Out-patient department. In each case one biopsy was taken at the first attendance. The patient was then instructed to brush his gums vigorously according to a given plan. He was provided with a standard brush and supply of dentifrice. Further specimens were taken after 1, 2 and 3 months of treatment.

Table 1

		1st biopsy	2nd biopsy	3rd biopsy	4th biopsy
Young	Mean	7·8	11·6 (48·0)	12·0 (53·8)	12·8 (64·1)
	S.E. of mean	0·48	0·44	0·48	0·34
Old	Mean	9·4	12·0 (27·5)	14·2 (51·0)	14·6 (55·3)
	S.E. of mean	0·47	0·69	0·87	1·2

The specimens were fixed in formalin, embedded in paraffin wax, sectioned perpendicularly to the epithelial surface, and stained in haematoxylin and eosin. The thickness of the cornified layer was then measured with a screw micrometer eyepiece using an oil-immersion objective. In all, 93 cases were studied; 48 'young', i.e. up to 30 years of age, and 45 'old' 31 years and over; at the fourth biopsy these numbers were reduced to 21 and 24 respectively.

Table 1 gives the thickness of the cornified layer for the four biopsies. It is the arithmetic mean of all the figures obtained in that age group. The units are microns. The figures in parentheses in columns 2, 3 and 4 are the percentage increments over column 1.

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THE RELATION OF POTASSIUM DEFICIENCY TO MUSCULAR PARALYSIS BY INSULIN

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It is known that patients treated for diabetic coma with large amounts of insulin and replacement of fluid may develop generalized paralysis of the extremities and of the respiratory muscles. During the paralysis the potassium level of the serum is extremely low, but when the serum potassium is raised by administration of potassium the paralysis disappears (Holler, 1946; Frenkel, Groen & Willebrands, 1947*a*, *b*; Nicholson & Brauning, 1947; Logsdon & McGavack, 1948; Tuynman & Wilhelm, 1948; Stephens, 1949; Danowski, Peters, Rathburn, Quashnock & Greenman, 1949).

It seemed possible that the paralysis might be explained by a combination of potassium depletion with an effect of insulin on the potassium distribution in the muscles, and further that the paralysis might be related to a diminution of the extra- or intracellular potassium or both. To test these possibilities we examined the effect of insulin on the contractions of the isolated rat diaphragm suspended in a potassium-free medium. The diaphragm preparations were obtained from normal rats and from rats fed on a potassium-free diet to produce a low intracellular potassium content of the muscle.

METHODS

Male white rats of Wistar strain were used from the time they weighed 60–90 g. Some were fed on a potassium-free diet, others, the control rats, received the same diet with additional potassium in the form of K_2HPO_4 3·35 g/kg mixed food. Gardner's diet slightly modified to the following composition was used. One kilogram of mixed food consisted of (g): sugar 725, casein 180, arachin oil 50, cod-liver oil 5 and salt mixture 40. The composition of the salt mixture was as follows (g): $CaCo_3$ 670, $CaHPO_4.2H_2O$ 119, NaCl 234, Na_2HPO_4 795, $MgSO_4.7H_2O$ 508, NaI 0·15, $CuSO_4.5H_2O$ 22, $ZnCl_2$ 0·4, $MnSO_4.4H_2O$ 16·2, and $FeNH_4$-citrate 148. In addition, the following amounts (mg) of B vitamins were added to each kilogram of mixed food: thiamine 3, riboflavin 3, niacin 20, paraaminobenzoic acid 250, Ca-pantothenic acid 20, pyridoxine 2, inositol 100 and choline 1 g. Each rat received further 0·2 ml. of wheat germ oil per week.

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After being fed on these diets for at least 21 days the rats were killed by decapitation. Blood was collected and allowed to clot and then centrifuged. The hind leg muscles were removed. The serum and the hind leg muscles were then analysed for water content, sodium, potassium and chloride concentrations. In the muscles 'neutral' fat was determined as well. The water content was determined by estimating the loss of weight after drying at 105° C during 24–48 hr. Sodium and potassium were determined by flame photometry; chloride was determined as described by Schales & Schales (1941) and 'neutral' fat by the method of Cotlove, Holliday, Schwartz & Wallace (1951).

In the calculations of the distribution of the electrolytes in the intracellular and extracellular spaces of the muscle, the chloride space was assumed to be identical with the extracellular space. Thus, by dividing the quantity of chloride per kg fresh fat-free muscle by the chloride concentration in the extracellular fluid, the quantity of extracellular water per kg fat-free muscle was obtained. A correction was made for the fat content of the tissue. For the calculation of the extracellular concentrations of chloride, sodium and potassium from the serum values a Donnan factor of 0·96 was used. By subtracting the extracellular quantity of water (in l./kg fresh fat-free muscle) from the determined water content of the whole muscle, the amount of intracellular water per kilogram of fat-free muscle was obtained.

By multiplying the extracellular concentration of sodium or potassium, by the amount of extracellular water, the quantity of extracellular sodium or potassium per kilogram fat-free muscle was found. By subtracting this figure from the *total* quantity of sodium or potassium per kilogram of fat-free muscle, the intracellular amounts of these electrolytes were obtained. The concentration of sodium or potassium in the intracellular water was then computed by dividing the total quantity of sodium or potassium by the quantity of intracellular water per kilogram of fat-free tissue (Westerink, Offerijns & Willebrands, 1955).

Rat diaphragm preparation. A phrenic nerve with the corresponding half of the diaphragm was suspended according to the technique described by Bülbring (1946) in a Tyrode solution in which the potassium concentration was varied. The bath volume was 100 ml., the temperature was 37° C, and a mixture of 95 % O_2 and 5 % CO_2 was bubbled through the solution. The nerve was stimulated with regular condenser discharges at a frequency of 12–16/min. The contractions of the diaphragm were recorded on a smoked kymograph.

RESULTS

Ion and water content of serum and muscle in rats fed on potassium-free diet

In the serum of rats fed on the potassium-deficient diet low blood levels of both potassium and chloride were found. In the muscles the intracellular sodium concentration was considerably increased and the intracellular potassium concentration was decreased (Table 1). For every 3 m-equiv intracellular potassium lost there was an increase in intracellular sodium of about 2 m-equiv. The chloride concentration in the muscles was not changed significantly. The amount of extracellular water in the muscles of the potassium-deficient animals was increased, whereas the amount of intracellular water was decreased by about the same amount.

Effects on the rat diaphragm

The diaphragms of the control rats contracted in Tyrode solution for hours without decrease in the height of the contractions. The potassium in the bath fluid could be diminished without greatly affecting the contractions. Even when potassium was entirely omitted from the Tyrode solution the diaphragms contracted for hours with only a small decrease in the height of the contraction (Fig. 1). The addition of 40 i.u. insulin to the bath fluid had no effect on the contractions of the diaphragms of the control rats, independent of whether they contracted in normal Tyrode solution (potassium content 2 m-equiv/l.) or in potassium-free solution (Fig. 1).

TABLE 1. Electrolyte changes brought about in rats fed on potassium-free diet. The values are means (+S.E.) obtained from 40 potassium-deficient and 12 control rats

	Potassium-deficient rats	Controls	*P*
Serum (m-equiv/kg H_2O)			
Na	150 ± 6·4	152 ± 3·1	>0·1
K	3·95 ± 0·6	7·15 ± 0·6	≪0·001
Cl	94 ± 6·7	118 ± 3·5	≪0·001
Muscle (m-equiv/kg fresh fat-free tissue)			
Na Total	50 ± 5·7	22·5 ± 3·3	≪0·001
Na Extracellular	26·5 ± 6·1	19·5 ± 2·6	≪0·001
Na Intracellular	24·5 ± 7·0	3 ± 1·9	≪0·001
K Total	68 ± 6·1	104·5 ± 7·1	≪0·001
K Extracellular	0·7 ± 0·17	0·9 ± 0·14	≪0·001
K Intracellular	66·1 ± 6·2	103·4 ± 7·2	≪0·001
Cl	17 ± 3·6	15 ± 2·4	>0·05 <0·1
Na Intracellular (m-equiv/kg H_2O)	41·5 ±10·5	5 ± 3·1	≪0·001
K Intracellular (m-equiv/kg H_2O)	112·5 ±12·0	163 ±12·1	≪0·001
Muscle water (ml./kg fresh fat-free tissue)			
Total	771 ± 8·8	765 ± 4·4	<0·05
Extracellular	182 ±34·0	129 ±18·7	≪0·001
Intracellular	590 ±37·4	636 ±18·5	≪0·001

Fig. 1. Contractions of rat diaphragm suspended in 100 ml. potassium-free Tyrode solution to stimulation of the phrenic nerve. Contractions after stimulation for 30 min (at A) and for 150 min (at B). A few minutes later (at C) 40 i.u. insulin added to the bath (at the arrow). Time in minutes.

The diaphragms of potassium-deficient rats contracted in ordinary and potassium-free Tyrode solution in the same way as the diaphragms of the control rats. Insulin had no influence on the contractions of these diaphragms when they were suspended in normal Tyrode solution, but it impaired their contractility when they were suspended in potassium-free Tyrode solution.

In thirty-three experiments of this kind, 40 i.u. insulin was added to the potassium-free fluid when the diaphragms of the potassium-deficient rats had contracted to a constant height for 30 min. In eleven of these experiments the insulin caused complete paralysis within a few minutes (Fig. 2); in nineteen it caused a great reduction in the height of the contractions (Fig. 3), and in three there was no effect. During the paralysis the diaphragms were relaxed.

Fig. 2. Contractions of rat's diaphragm suspended in 100 ml. Tyrode solution. Rat fed on potassium-free diet. At A paralysis after adding 40 i.u. insulin at arrow; at B return of muscular contractions after adding 20 mg KCl; C, 5 min later. Time in minutes.

In this condition direct stimulation of the diaphragm caused no contraction. The effect of insulin was usually tested by adding 40 i.u. (2 mg) to the 100 ml. bath, but the addition of 0·2–2 i.u. (10–100 μg) was sufficient to produce a reduction in the height of the contractions.

The effect of insulin could be abolished by adding KCl to the bath fluid (Fig. 2B and Fig. 3B). Similarly the addition of KCl to the bath fluid before the addition of insulin prevented the paralysing effect.

The addition of purified human serum albumin to the potassium-free solution did not influence the contractions of the diaphragms from rats fed on

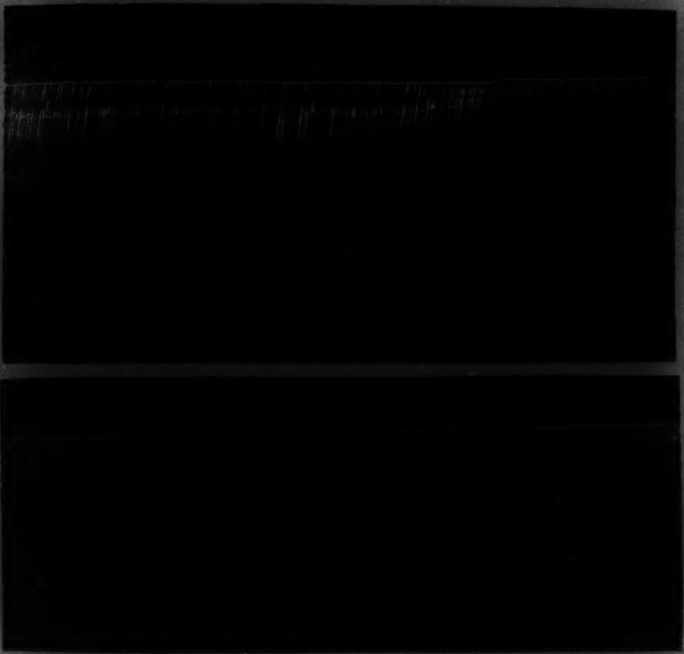

Fig. 3. Contractions of rat's diaphragm suspended in 100 ml. Tyrode solution. Rat fed on potassium-free diet. A, effect of 2 mg albumin and of 40 i.u. insulin; B, effect of 20 mg KCl. Time in minutes.

TABLE 2. Mean concentrations of Na^+, K^+ and Cl^- in 28 potassium-deficient rats and in 12 normal rats, arranged according to the effect of insulin on the contractions of the diaphragm *in vitro*

			Extracellular fluid (m-equiv/kg H_2O)			Intracellular concentrations (muscle)			
						(m-equiv/kg fresh fat-free tissue)		(m-equiv/kg H_2O)	
Group	Effect of insulin	No. of animals	Na^+	K^+	Cl^-	Na^+	K^+	Na^+	K^+
I	Paralysis	8	152	3·63	90·9	24·8	65·6	42·6	113·5
II	Contraction height diminished	17	150	3·98	94·6	21·6	68·3	36·6	117·8
III	No effect	3	149	4·6	98·0	26·3	75·3	43·2	124
Normal (controls)	No effect	12	152	7·15	118	3	103·4	5	163

a potassium-free diet, nor did it abolish the paralysing effect of insulin (Fig. 3).

The mean concentrations were determined of Na^+, K^+ and Cl^- in the serum and in the muscles from twenty-eight potassium-deficient rats. In Table 2 these values have been arranged in three groups. It is evident that insulin produced full paralysis in those rats which were most deficient in potassium. In those rats which were somewhat less deficient in potassium, insulin produced only a decrease in the height of the contraction, and in the three animals in which potassium deficiency was least pronounced insulin had no influence on the contractions. There was no correlation of the insulin effect with the sodium content of the muscles.

DISCUSSION

The electrolyte concentrations which we found in the normal and in the potassium-deficient rats are of the same order as those obtained by previous workers (Miller & Darrow, 1940; Darrow, Schwartz, Iannucci & Corille, 1948; Gardner, Talbot, Cook, Berman & Uribe, 1950; Cotlove *et al.* 1951). Only the values for the extracellular potassium concentration of the control animals were somewhat higher (7 m-equiv/kg) than those of most investigators (5 m-equiv K^+/kg) probably owing to the fact that we did not heparinize the rats before sampling the blood and that during coagulation the erythrocytes may lose considerable amounts of potassium.

Our findings on the rat diaphragm preparation, which showed that neither extracellular nor intracellular potassium deficiency *per se* causes paralysis of the muscle, agree with those of Miller & Darrow (1940) who could find no abnormality in the contractility of the gastrocnemius muscles from potassium-deficient rats. In man also a potassium-free diet does not cause paralysis (Black & Milne, 1952; Fourman, 1954; McArdle, 1954) nor does a low concentration of extracellular and intracellular potassium caused by pathological loss of potassium. Thus potassium deficiency alone is insufficient to produce paralysis *in vivo* or *in vitro*. On the other hand, our finding that insulin produces paralysis of the diaphragm obtained from rats fed on a potassium-deficient diet and suspended in potassium-free Tyrode solution shows that insulin has a paralysing effect on the potassium-deficient muscle and this action of insulin may well explain the paralysis which can occur when patients with diabetic coma are treated with insulin. This effect of insulin does not necessarily depend on the extracellular or intracellular potassium concentration alone; other factors of the 'potassium deficiency complex', like the altered sodium and chloride concentrations, the changes in water content, pH, etc., may contribute to it.

The paralysing effect insulin exerts on the potassium-deficient diaphragm illustrates that even a hormone which has been studied as extensively as insulin reveals, when tested during a disturbed electrolyte balance, unexpected 'new' properties. The same may apply to other drugs; for instance, it is known that the action of digitalis is counteracted by high concentrations of potassium in serum and accentuated by low concentrations (Cohen, 1952). Since many drugs are used in patients with disturbed electrolyte metabolism, it seems necessary that they should be investigated not only in normal animals, as is customary, but also in animals with disturbances in their electrolyte equilibrium similar to those that occur in human patients.

SUMMARY

1. Insulin exerts a paralysing effect on the isolated diaphragm preparation of rats fed on a potassium-free diet and suspended in potassium-free Tyrode solution.

2. This action of insulin may explain the paralysis which may develop in patients with diabetic coma treated with large doses of insulin.

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CONTRACTION IN MOLLUSCAN SMOOTH MUSCLE

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There are several invertebrate somatic smooth muscles, some as long as 5 cm, in which parallel fibres extend for at least half the length of the muscle. Such smooth muscles are very suitable for study with the modern techniques that have been so successfully used in recent work on the mechanical and thermal properties of frog skeletal muscle.

In comparison with mammalian visceral smooth muscle, the functions of the molluscan muscles resemble more closely those of vertebrate skeletal muscle. But both types of smooth muscle are clearly distinguished by their ability to maintain considerable tension for very long periods of time. In the case of lamellibranch adductor muscles durations of 2–5 days are common (cf. Lowy, 1953).

This paper describes the first results of investigations which have been started with the object of discovering what structural and physiological features of molluscan smooth muscles are responsible for their outstanding tonic properties.

METHODS

The following muscle preparations have been used: the anterior byssus retractor muscle (ABRM) and the pedal retractor muscle (PR) of *Mytilus edulis*, and the retractor of the pharynx of *Helix pomatia*. The *Mytilus* muscles were tied at both ends and cut away from their attachments; aerated sea water was used as a bathing medium. The dissection of the *Helix* muscle has been described by Ramsay (1940) and the composition of its bathing solution by Abbott & Lowy (1957).

The muscles were mounted on a multi-electrode stimulating assembly (Hill, 1949) with silver wires spaced at 3 mm intervals providing alternate anodes and cathodes along the length of the muscle. Stimulation was either by condenser discharges or by square wave pulses. One end of the muscle was firmly clamped and the other connected by a light silver chain to the recording apparatus. Isometric tensions were registered by an isometric lever with a shadow activating a photocell or by an RCA 5734 transducer. Display was on a cathode-ray tube. Isotonic contractions were studied with an afterloaded isotonic lever and photo-electric recording of movement.

For each type of muscle the relation was first determined between strength and duration of stimulation, using the smallest detectable rise in tension to indicate threshold.

Mechanical and thermal events during stimulation with repeated shocks were studied. Isotonic conditions provided information about the speed of shortening of the muscles under different loads. Constant speed stretches or releases were applied during isometric contraction by means of a Levin–Wyman ergometer and the tension changes were recorded during and after movement.

Heat measurements were made using a thermopile feeding into a galvanometer–photocell amplifier (Hill, 1948). The ABRM was divided along its length and one half laid on each side of the thermal element; with the *Helix* muscle there was tissue on one side of the thermopile only. Heat changes were measured when the muscles were stimulated with repeated shocks under isometric conditions at various muscle lengths as well as during isotonic contractions. Some measurements have also been made of the heat production during prolonged contractions of the ABRM caused by application of acetylcholine (ACh) (which induces a 'tonus') and of the changes when relaxation was caused by 5-hydroxytryptamine (5-HT) (which in *Mytilus* muscle acts as a relaxing agent (Twarog, 1954)). In these experiments the muscle on the thermopile was connected to a reservoir containing sea water which was kept in the same Dewar vessel as the thermopile chamber. When the system was in equilibrium, the sea water could be blown carefully into the chamber to cover the muscle and then run back into the reservoir without appreciably disturbing the thermopile balance. ACh was run into the water-bath and allowed to equilibrate in temperature with the sea water. The sea water containing the ACh was then forced into the muscle chamber and caused contraction. After about 15 sec the fluid was run out again and the thermal changes measured for up to half an hour. There were considerable difficulties in these experiments because of the slow steady changes in osmotic pressure in the muscle even at rest, but the resultant temperature differences were not large enough to mask the increased heat production during tonic contraction.

RESULTS

Responses to single shocks

All the muscles studied responded to a single electrical shock and the strength–duration curves were found to be very similar. For muscles in good condition, a suitable threshold stimulus was found to be about 2 V with a pulse width of 2 msec (cf. Fig. 1, Abbott & Lowy, 1956*a*).

PR of Mytilis. On stimulation with single shocks of increasing intensity this muscle responded with twitches which were found to increase up to a maximum. Provided a moderate pulse width was used (say 2 msec), this maximum appeared to persist even when the intensity was further increased. However, when a long pulse was used the muscle often responded by repetitive discharges. This type of response is characterized by a slower continued rise in tension beyond the peak of the normal twitch, and is sometimes followed by spontaneous rhythmic activity.

ABRM of Mytilis and pharynx retractor of Helix. The existence of maximal twitches has been claimed in the *Helix* muscle (Bozler, 1930) and denied in the ABRM (Schmandt & Sleator, 1956). On this issue we do not consider our experiments to be conclusive because of the possible damage to the muscles with very strong shocks. But we found that in every instance the time taken to reach 'peak' tension was much shorter than the relaxation time. Thus, in the *Helix* and PR muscles peak tension at 14° C was reached within 200–250 msec, whereas during relaxation tension decayed to half-value in 1·3–1·5 sec. The corresponding events in the ABRM take 1–1·5 sec and 4–6 sec.

In the case of the PR muscle, measurements were made of the speed at which the mechanical events are propagated along the length of the muscle. The method used for this experiment has been described by Abbott & Ritchie (1951). Unloaded isotonic twitches obtained with stimulation at one end only were compared with those resulting from all-over multi-point stimulation. The amount of shortening in the two types of twitches was almost the same (Fig. 1), and this suggests that excitation is propagated along the whole length of the muscle fibres. The time displacement between the two curves represents the time taken for the excitation to travel half the length of the muscle. In this case the time was 24 msec for a 19 mm length of muscle on the electrodes, i.e. a propagation velocity of 40 cm/sec at 14° C.

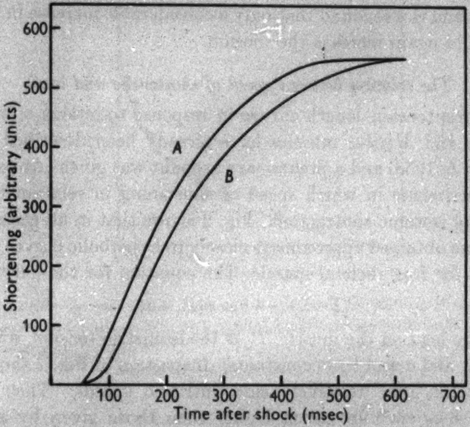

Fig. 1. *Mytilus* pedal retractor muscle. Shortening in unloaded isotonic twitches. Curve *A*, simultaneous stimulation all over; *B*, stimulation at one end. Temp. 14° C.

Responses to repetitive stimulation

In all the muscles repeated shocks induce mechanical responses which sum like a tetanus. Fusion of the mechanical responses occurs at a repetition frequency of 5–8 shocks/sec for the PR and *Helix* muscles, and 2–4 shocks/sec for the ABRM. But whereas below the fusion frequency in the PR of *Mytilus* (and in the *Pinna* fast adductor, cf. Abbott & Lowy, 1956*a*) the tension rise in response to the second shock was found to be almost the same as to the first, there is evidence for mechanical facilitation in the other two muscles. Thus, in a typical experiment where the ABRM was stimulated below fusion frequency, the tension changes in response to the first three shocks were 2, 6·5 and 9 g, respectively.

All muscles gave consistent maximum contractions when stimulated above tetanus fusion frequency. Under isometric conditions, the tensions were considerably greater than the tensions obtained in response to single shocks: about 2–3 times in the PR, 5–7 times in the *Helix* muscle, and 8–10 times in the ABRM.

In the case of the ABRM an attempt was made to produce prolonged isometric contraction by repetitive stimulation. But it was found that under these conditions of excitation tension begins to drop if stimulation is continued for longer than 2–4 min. We thought at first that this could be explained by assuming that the muscle had become more economical and that the stimulation frequency had become too high. We therefore tried to maintain tension using progressively decreasing frequency of stimulus. This was also unsuccessful, and it was found that only a considerable increase in stimulation intensity would again increase the tension.

The relation between speed of shortening and load

The isometric tension–length curves in response to tetanic stimulation for *Pinna*, *Helix* and *Mytilus* muscles have already been described (Abbott & Lowy, 1956*a*, *b*, 1958) and a preliminary account was given (Abbott & Lowy, 1953) of experiments in which speed of shortening in relation to load was studied during isotonic contraction. Fig. 2 shows that in all cases the force–velocity curves obtained approximate closely to hyperbolic curves such as Hill (1938) found for frog skeletal muscle. The equation for the curve is usually given as

$$(P+a)\,(v+b)=b(P_0+a),$$

where P is the load on the muscle, P_0 is the isometric tension, v is the speed of shortening, and a and b are constants. Inspection of Fig. 2 shows that the smooth muscles have relatively slow intrinsic speeds. Their maximum tensions (3·5–8 kg/cm^2) are much higher than those given by a variety of striated muscles (cf. Wilkie, 1954).

Plasticity in active muscle

In order to obtain further information about the mechanical properties of the contractile elements during isometric repetitive stimulation a study was made of tension changes after stretch or release of the active ABRM, using the Levin–Wyman ergometer. It was found that if the release was very rapid there was appreciable redevelopment of tension, whereas after a slow shortening the tension remained considerably below isometric value (Fig. 3). In a similar way, the changes of tension following stretches of the active muscle at constant speed were studied and here it was found that the quicker the stretch the less was the final excess above the isometric tension (Fig. 4). Similar results have been obtained with vertebrate striated muscles (Abbott & Aubert, 1952), but the deviation is much more pronounced in the molluscan smooth muscles.

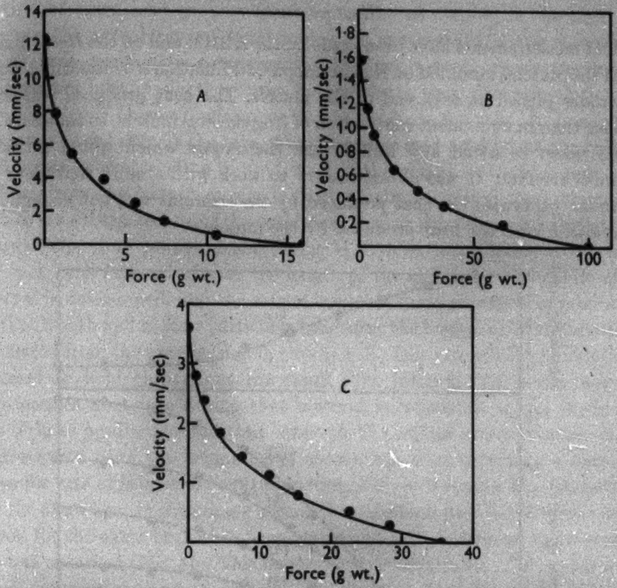

Fig. 2. Force–velocity curves. Values for a and b calculated by plotting P against $(P_0 - P)/v$. *A*, *Mytilus* pedal retractor, $a = 2{\cdot}2$ g ($a/P_0 = 0{\cdot}14$), $b = 1{\cdot}7$ mm/sec (0·094 length/sec); *B*, *Mytilus* ABRM, $a = 20$ g ($a/P_0 = 0{\cdot}2$), $b = 0{\cdot}33$ mm/sec (0·01 length/sec); *C*, *Helix* pharynx retractor, $a = 11$ g ($a/P_0 = 0{\cdot}3$), $b = 1$ mm/sec (0·045 length/sec). Temp. 14° C.

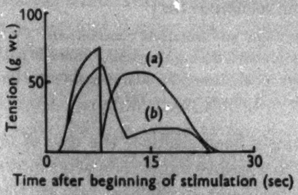

Fig. 3

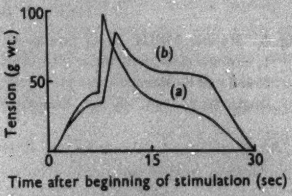

Fig. 4

Fig. 3. *Mytilus* ABRM. Recovery of tension during isometric contraction after shortening (30–27 mm) at (*a*) 25 mm/sec; (*b*) 1 mm/sec. (Copy of photographic record.)

Fig. 4. *Mytilus* ABRM. Tension changes during isometric contraction following stretch (27–30 mm) at (*a*) 5 mm/sec; (*b*) 0·5 mm/sec. (Copy of photographic record.)

Heat production

Heat measurements have been made on the ABRM and on the *Helix* muscle. With the muscles mounted on the thermopile, stimulation is necessarily through electrodes placed at each end of the muscle. The heat produced was much smaller than in equivalent contraction of frog skeletal muscle, so that care had to be taken to avoid any leak in the thermopile which would introduce stimulus artefact. It was also necessary to work with stimuli that were just maximal, otherwise the heat produced by the stimulus was not negligible in comparison with the heat produced by the muscle.

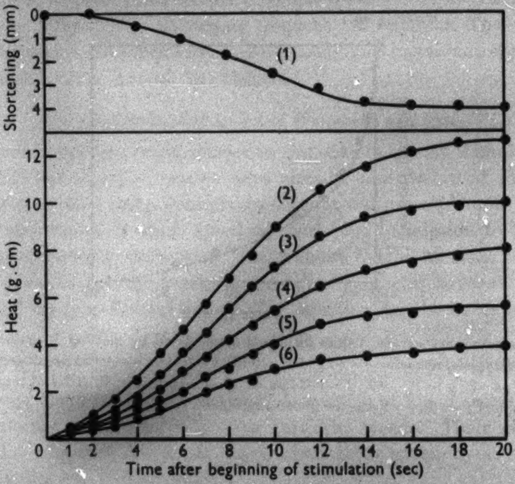

Fig. 5. *Mytilus* ABRM. Heat production during isotonic and isometric contractions. Curves: 1, Isotonic shortening under 26 g load; 2, heat production during shortening under 26 g load; 3, 4, 5 and 6, isometric heat production at lengths 26, 25, 24 and 22 mm, respectively. Initial length 26 mm, wt. 130 mg. Stimulation for 14 sec, 2 shocks/sec, at 14° C.

As in striated muscles that have previously been studied, the rate of heat production under isometric conditions fell to a steady value soon after internal shortening was completed. But the values for maintenance heat rate are much smaller in the smooth molluscan muscles. Thus, the ABRM at 14° C produces about 0·1 mcal/g.sec, compared with 1·5 mcal/g.sec in frog sartorius muscle at 0° C (Abbott, 1951).

When isotonic shortening is allowed, extra heat is produced (Fig. 5, curve 2). Estimates of the amount of heat produced during shortening are complicated by the fact that the maintenance heat varies with length. In the definition of the extra heat of shortening, the assumption is made that the rate of production of extra heat at any time during the shortening is the difference between the actual rate of heat production measured and the rate of isometric heat production corresponding to that instant. Since the rate of maintenance heat production varies both with the time after stimulation and with length (or tension), allowance has to be made for this. In our experiments we have taken as the rate of maintenance heat production the value at each instant corresponding to the length of the muscle at that instant.

Thus, from the instant at which shortening is permitted the maintenance heat to be subtracted from the total heat during shortening is calculated as follows. The time course of shortening is divided into blocks of 250 msec. For each such interval of time the length of the muscle at the middle of the interval is determined, and the rate of isometric maintenance heat production for that length and at that particular time after the beginning of stimulation is calculated from the graphs (Fig. 5, curves 3–6). This rate of heat production is assumed to hold throughout the small time interval (250 msec), and the maintenance heat rate during that interval is calculated as the product of rate of heat production and time interval. The values obtained are summed progressively from the beginning of shortening, thus providing a corrected curve for rate of isometric heat production. This is shown in Fig. 6*a*, curve 3, and the extra heat in shortening is then the difference between the two curves. Values for the extra heat have been plotted against shortening and a straight line was obtained (Fig. 6*b*). This method of calculation has of necessity introduced assumptions and approximations, but the linearity of the resulting relationship would seem to justify the procedure. In frog skeletal muscle it is well known that there is a linear relation between extra heat and shortening.

An attempt has been made to estimate heat production during tonic contraction induced by ACh. Heat production during prolonged repetitive electrical stimulation was measured first. For this the muscle was soaked in sea water and during heat measurements the water was run out into the attached reservoir. The required amount of ACh (usually to give a final concentration in terms of the salt of 1 μg/ml.) was then run down into the reservoir and (after equilibration) forced into the muscle chamber as described under Methods. Tension began to rise within a few seconds of beginning ACh treatment, reached a maximum within about 30 sec, and slowly declined during the following half an hour or so. Previous experiments showed that when equilibrated sea water was run in or out there were few or no thermal disturbances and any changes were over within a few seconds. After the addition of ACh a large positive heat deflexion was always observed, associated with the tonic contraction.

It proved impossible to obtain very accurate measurements of the heat production during the ACh tonus. The heat loss of the rather fast thermopiles that were used complicated the observations, and in addition there was always the possibility that changes of osmotic pressure in the muscle, which produce vapour pressure and thermal changes, might interfere with the determinations. It was in fact found that if a freshly dissected ABRM was left in air on the thermopile there appeared a steady rise of voltage output across the thermopile over a period of several hours. This was probably due to changes in osmotic pressure within the muscle. Dr W. Potts (personal communication) has observed similar changes in osmotic pressure of the ABRM by direct measurements.

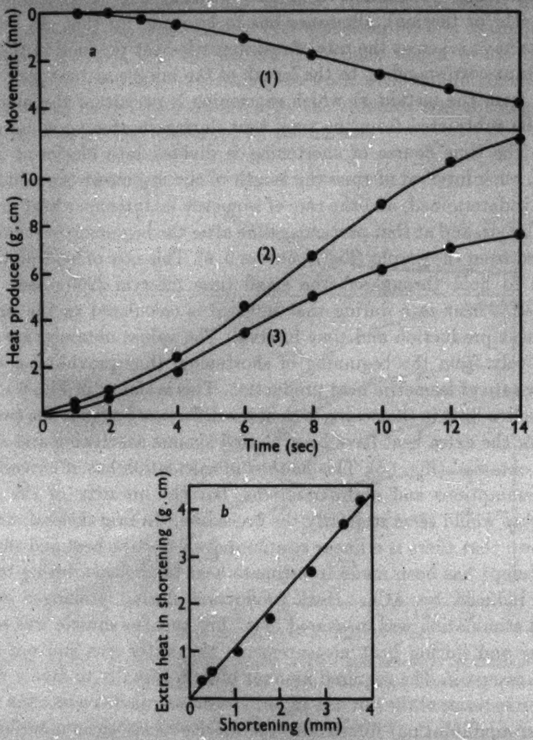

Fig. 6. *Mytilus* ABRM. Same experiment as for Fig. 5. Curves: (*a*) 1, Isotonic shortening; 2, isotonic heat production; 3, isometric heat production (for details see text). (*b*) Relation between distance shortened and heat produced in shortening.

The heat due to such changes of the muscle was always small compared with the changes during ACh tonus and this observation, together with the fact that the large positive production of heat can always be abolished when the muscle is relaxed by treatment with 5-HT, convinced us of the reality of increased heat production during ACh tonus.

Relaxation in the PR of Mytilis

This muscle was chosen for closer study as it consistently showed complete relaxation either following a single shock or after repetitive stimulation. When the time course of the decay of active state was followed by the method of Ritchie (1954) it was evident that, as in frog skeletal muscle (Ritchie & Wilkie, 1955), active state disappears before tension decay is complete. The experiment was done by allowing a small rapid release of the muscle at various times along the relaxation curve and recording the tension redeveloped: the peak of each such tension record lies on the active-state curve (Ritchie, 1954). Fig. 7 shows the results of such an experiment where releases were permitted during relaxation following an isometric twitch of the *Mytilus* PR. The time taken to reach maximum twitch tension is about 250 msec; active state disappears by 1·5 sec; yet tension does not drop to zero for another 4·5 sec.

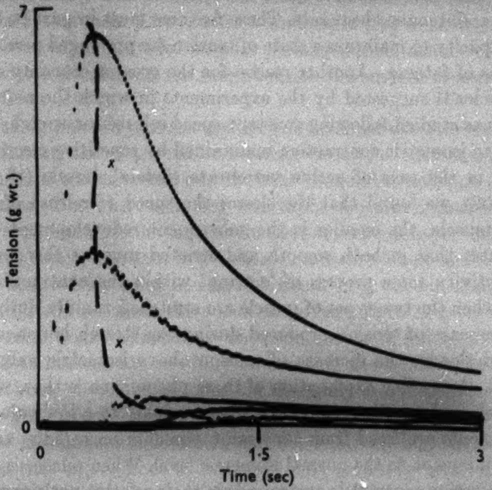

Fig. 7. *Mytilus* pedal retractor. Active-state curves obtained from experimental tension–time curves. Curve (x) drawn through peaks of tension–time curves shows decline in intensity of active state. Temp. 14° C.

In experiments on frog skeletal muscle at 0° C, Ritchie & Wilkie (1955) found the time to reach maximum twitch tension to be about 250 msec. But here active state has practically disappeared by 400 msec and tension has fallen to zero by about 650 msec. It would appear that in comparison with frog muscle the smooth muscle can maintain tension for a considerably longer time after it has ceased to be able to develop tension actively.

DISCUSSION

Compared with frog skeletal muscle, the smooth molluscan muscles studied here are slow in their relaxation and velocity of shortening and have a remarkably small maintenance heat rate. These features must in part be responsible for their capacity to maintain a state of tension for prolonged periods without visible signs of fatigue. Another reason for the greater economy of the molluscan muscles is suggested by the experiments in which the redevelopment of tension was studied following constant-speed releases or stretches along the plateau of an isometric contraction maintained by repetitive electrical stimulation. As in the case of active vertebrate skeletal muscle (cf. Abbott & Aubert, 1952), we found that the slower the speed of release of the active molluscan muscle, the smaller is the subsequent redevelopment of tension. This indicates that in both smooth and striated muscles there occurs as a result of activity some process of 'setting' within the contractile material. Similarly, when the two types of muscle are stretched rapidly during activity the large increase of tension produced during the stretch is not maintained. If stretched slowly, the increase of tension above isometric value is better maintained. A possible explanation of these phenomena is that, when active muscle is released rapidly and tension drops quickly to a low value, the contractile elements are freed from constraint, can shorten rapidly, and proceed to redevelop tension to the normal isometric level. When release is slow, some parts of the active contractile system retain their relative positions and at the end of release cannot contribute towards the complete redevelopment of tension. During rapid stretch some disruption of the active pattern may occur, whereas if stretch is slow some of the contractile material may be strained while keeping its active, organized configuration, so that a final tension in excess of the normal isometric value is obtained. Similarly, when the contractile material is stretched during normal relaxation the strain due to the displacement of the 'set' pattern might account for the fact that tension outlasts decay of active state.

The experiments with the PR of *Mytilus* demonstrate clearly that the muscle can maintain tension after active state has decayed for much longer than frog muscle. This, together with the observation that after a slow stretch of the active ABRM of *Mytilus* the tension increase above isometric value is more pronounced than in frog muscle, strongly suggests that there is a significant difference between the two types of muscle in the extent of 'setting' within the contractile material which occurs as a result of activation. But the idea that there is a greater extent of 'setting' in the smooth muscles does not imply the operation of a 'catch' mechanism which makes possible the maintenance of tension without any expenditure of energy. It merely means that, compared with frog muscle, tension in the smooth muscles persists for longer after stimulation has ended and active state has decayed. In the absence of further re-activation tension steadily decays. Therefore, the maintenance of a steady level of tension necessitates continuous excitation. Intermittent excitatory bursts in localized areas can readily be demonstrated in smooth muscles of intact animals (Lowy, 1953). And, as in frog muscle, yet another measure of economy is provided because prolonged maintenance of tension is associated with a decrease in the rate at which the smooth muscle has to be re-excited (Lowy, 1953).

The molluscan smooth muscles and frog skeletal muscle also resemble one another in showing the same relation between velocity of shortening and load, and between distance shortened and the heat liberated in addition to the heat of maintenance. But whereas in frog muscle the constant *a* derived from the mechanical experiments is the same as the heat of shortening per unit distance, this is not apparently the case in the smooth muscles. Thus, in the ABRM of *Mytilus* the heat of shortening measured is about 11 g.cm/cm, compared with values for *a* derived mechanically which range from 19 to 23 g.cm/cm. However, as the conditions for initiation and maintenance of full activity in these isolated smooth muscles are not fully understood, the significance of the discrepancy between the values of *a* derived thermally and mechanically cannot be assessed. For instance, in the ABRM the absence of maximal twitches, the possibility of mechanical facilitation, and the relatively high tetanus: twitch ratio suggest that active state is developed only gradually after stimulation.

Nor is it possible at this stage to discuss the possible reasons why repetitive electrical stimulation fails to maintain tension in the ABRM for prolonged periods. Winton (1937) found that a contraction which may last for 30 min or longer can be induced in this muscle by a d.c. stimulus. He suggested that here the observed very slow relaxation is due to a greatly increased muscle viscosity. No attempt has been made in the present experiments to test this hypothesis.

The phenomenon of tonic contraction induced in the ABRM by ACh requires some discussion in view of Twarog's (1954) claim that it demonstrates the existence of a 'catch' mechanism because contraction outlasts depolarization and 5-HT brings about relaxation although depolarization persists. Twarog concludes, 'continued depolarization, that is, excitation, is not required for maintenance of the contraction'. It is true that in a muscle tonically contracted with ACh relaxation is greatly delayed, but electrical activity, i.e. excitation, due to discharges from muscle fibres, is present throughout the time tension is maintained (Hoyle & Lowy, 1956) together, as was shown here, with increased heat production.

The mechanism of ACh tonus may be explained as follows. On application of ACh the muscle is depolarized and contraction results. Once the ACh is washed off, the muscle membranes begin to repolarize, but nerve or muscle elements continue to give spontaneous discharges (cf. Hoyle & Lowy, 1956). Because of the random nature of this electrical activity its contribution to the measured steady depolarization is small. As the spontaneous firing rate declines, tension also decays gradually. Thus, as found by Twarog (1954), tension outlasts depolarization.

Tension can be reduced rapidly by treatment with 5-HT as well as by inhibitory stimulation applied at the entry of the nerve into the muscle (Hoyle & Lowy, 1956). Twarog (1954) showed that relaxation induced by 5-HT is not accompanied by repolarization, indicating that the drug may exert its effects directly on the contractile substance. It is known that in the crustacean nerve–muscle system one type of inhibition induces relaxation without affecting the muscle action potentials (Marmont & Wiersma, 1938). That a similar mechanism may operate in lamellibranch molluscs has already been suggested from experiments with intact animals (Lowy, 1953). Actually relaxation with 5-HT of an ACh-contracted isolated ABRM of *Mytilus* is often accompanied by increased electrical activity (Abbott & Lowy, unpublished results). This could explain Twarog's findings without the assumption of a 'catch' mechanism. The issue concerning the action of 5-HT might be resolved by an experiment in which a glycerol-extracted ABRM, contracted by adenosinetriphosphate, is treated with 5-HT.

No explanation can be given at present for the observation that the molluscan smooth muscles are able to develop a greater maximal tension per unit cross-section than any other type of muscle studied hitherto.

SUMMARY

1. Certain mechanical and thermal events during activity of long-fibred smooth molluscan muscles have been studied.

2. In all cases the speed of shortening is related to load during isotonic contraction in a manner approximating closely to Hill's (1938) characteristic equation.

3. Shortening of stimulated muscle is accompanied by extra heat production proportional to shortening. The constant a for shortening heat differs from the value a in the characteristic equation.

4. Redevelopment of tension following release of muscle during maintained contraction decreases with the speed of release. Fall of tension after stretch during maintained contraction also decreases with speed of stretch.

5. Relaxation of tension is very much slower than rise of tension at the onset of contraction and the active state is over long before tension in relaxation drops to zero.

6. The ability of the muscles to maintain tension economically is discussed in terms of their slowness of relaxation, their small maintenance heat rate and the extent of 'setting' within the contractile material as a result of activity. Tension is assumed to be maintained by spontaneous random re-excitation of the muscle rather than by a special 'catch' mechanism.

We wish to thank the Director of the Plymouth Laboratory for his interest and encouragement. Part of the electrical apparatus was purchased with grants from the Royal Society.

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MECHANICAL PROPERTIES OF *HELIX* AND *MYTILUS* MUSCLE

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Tension can be produced in muscle by excitation (active tension), by relatively slow stretch of resting muscle beyond maximum body length (resting tension), or by rapid stretch of resting muscle (which produces a transient tension, cf. Abbott & Lowy, 1957). In this paper we consider the conditions in which two types of long-fibred molluscan smooth muscle produce resting tension, as well as the relation between stress relaxation (decay of transient tension) and the fall of active tension following isometric contraction. These phenomena have been given particular attention because in the course of this investigation it very soon became apparent that the results differed significantly from those obtained by Bozler (1930, 1931) who worked with the same smooth muscles. Bozler's emphasis on the absence of resting tension at muscle lengths below *reference length* (longest length in the animal, cf. Abbott, 1951) appears to have diverted attention from the resemblances to striated muscle which become evident when greater lengths, at which we find that true resting tension appears, are also taken into consideration. In the case of transient tension, Bozler's experiments showed that it decayed along the same exponential time course as relaxation from isometric contraction. Bozler therefore concluded that 'in the beginning of relaxation, (smooth) muscle is in the same physical state as if the resting muscle had been stretched' (Bozler, 1936*a*; p. 429). But this view cannot be accepted because, with refined recording techniques, we find that the time course of stress relaxation always differs from the time course of relaxation after an isometric tetanic contraction: the two phenomena are not identical in the molluscan smooth muscles any more than in striated muscle (cf. Hill, 1950).

METHODS

Two smooth muscle preparations have been used: the anterior byssus retractor of *Mytilus edulis* (ABRM) and the retractor muscle of the pharynx of the snail *Helix pomatia*. The dissection of the ABRM was described by Hoyle & Lowy (1956), and that of the snail muscle by Ramsay (1940). The *Helix* muscle was bathed in a solution containing (g/l.) NaCl 3·5, KCl 0·15, $CaCl_2$ 0·5, $MgCl_2$ 0·3, and 5 mg P as phosphate buffer to bring it to pH 7·5. Sea water was used as a bathing solution for the ABRM.

The isolated muscle was mounted on a multi-electrode stimulating assembly (Hill, 1949) and connected by a light silver chain to an isometric lever. Resting or active tension was recorded either photo-electrically on a cathode-ray tube or mechanically on a smoked drum. The length of the muscle was altered by raising or lowering a Palmer stand. Condenser discharges were used to stimulate the muscle. An Ediswan pen-writing oscillograph recorded muscle action potentials.

After isolation the muscle was weighted with a load of 10–20 g to bring it to its longest length in the animal (*reference length*). Further extension of the muscle was limited by a stop. Maximal isometric tensions were then recorded at a number of lengths shorter than the reference length.

Starting again at reference length, the muscle was stretched by about 10 %, and after a 5 min interval resting and active isometric tensions were measured. This was repeated until the muscle had been stretched to about 75 % above its reference length.

5-Hydroxytryptamine (5-HT) was used to abolish tonic responses of the ABRM (Twarog, 1954; Hoyle & Lowy, 1956). The drug was dissolved in distilled water to give a concentration of 1 mg/ml. and this solution was diluted with sea water as required. The experiments on resting and active isometric tension were repeated, using the ABRM treated with 5-HT in order to eliminate any possible effect of spontaneous tonic activity.

In the snail muscle stress relaxation (tension decay following stretch of resting muscle) was also investigated, using methods described in another paper (Abbott & Lowy, 1957). The time course of stress relaxation was compared with that of relaxation following isometric tetanic contraction. The length of the muscle for the isometric tetanus was the same as that at the end of stretch for the stress relaxation. The effects of changes in temperature on the time courses of stress relaxation and of relaxation following isometric contraction were also studied.

RESULTS

Resting tension and the isometric tension–length curve

The *Mytilus* ABRM exerts no appreciable resting tension below reference length; above it a true resting tension appears. Whenever the length of the muscle is increased a tension is produced; if the final length is below reference length this tension is transient and decays to a negligible value. But if the final length is above the reference length, the transient tension falls to a value which is appreciable and reaches a reasonably steady level within about 5 min. This finding was used to determine the isometric tension–length curve. The active tensions at lengths below reference value were first measured. Then, starting from reference length, the muscle was progressively stretched and an interval of 5 min after each length increase was allowed before both resting and active tensions were measured. This procedure gives both the isometric tension–length and the resting tension curves (Fig. 1). Active isometric tension has a maximum value at about the longest length reached by the muscle in the animal and at this length appreciable resting tension begins to appear.

If tension in a muscle stretched beyond reference length is followed for longer periods it is found to decay, but extremely slowly, for days. At the same time, both the resting and active isometric tension–length curves move along the length axis (Fig. 1). This represents a 'slip' in the muscle and may correspond to the effect which has been observed when frog sartorius muscle is stretched to about 60% above its reference length (Aubert, Roquet & van der Elst, 1951).

Similar results have been obtained using the pharynx retractor of *Helix* (Abbott & Lowy, 1956*a*), the adductor of *Pinna* (Abbott & Lowy, 1956*b*), the pedal retractor of *Mytilus* and the smooth part of *Pecten's* adductor (Abbott & Lowy, unpublished results).

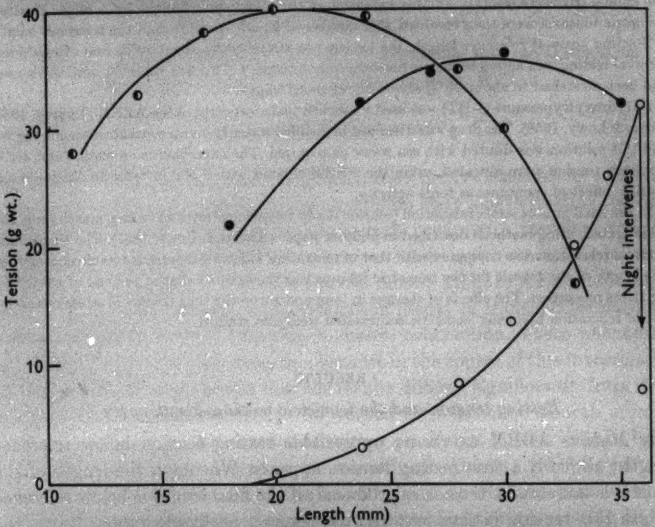

Fig. 1. Active and resting tension–length curves of the anterior byssus retractor muscle of *Mytilus*. ◐, Isometric tension–length curve at beginning of experiment (tension developed in excess of resting tension); ○, resting tension, measured 5 min after each increase of length—muscle then left to soak in aerated sea water during the night at 36 mm with resultant drop in resting tension; ●, isometric tension–length curve next day. Muscle weight 14 mg, temp. 14° C, reference length 20 mm.

The existence of true resting tension

The possibility that the tension produced by stretch of resting muscle beyond reference length is due to spontaneous tonic activity can be eliminated in the case of *Mytilus* muscle by the following experimental evidence.

(1) Application of 5-HT selectively abolishes the capacity for maintained tonic contraction (Twarog, 1954; Hoyle & Lowy, 1956). The treated muscle continues to give normal phasic responses and tetanic tensions. Above the length for maximum isometric tension, long-lasting tension still appears on stretch of the resting muscle (Fig. 2).

(2) Maintained tension, developed as a result of activation by ACh or d.c. stimulation, can be completely abolished by stimulation of inhibitory nerves (Hoyle & Lowy, 1956). In contrast, the steady tension resulting from stretch of the resting muscle beyond reference length is not affected by such stimulation (Fig. 3).

(3) Recovery of tension in active muscle after quick release is quite appreciable (Abbott & Lowy, 1958), whereas it is very small when the resting muscle is rapidly released—although the resting tension may have been as large as the maximum tension developed during activity.

Fig. 2. Effect of 5-HT (10^{-6} g/ml.) on resting tension of the ABRM. *T*=tetanus, condenser discharges 5/sec; *Str.*=stretch of resting muscle above reference length; *R*=release of muscle. Time marker 5 sec, temp. 14° C.

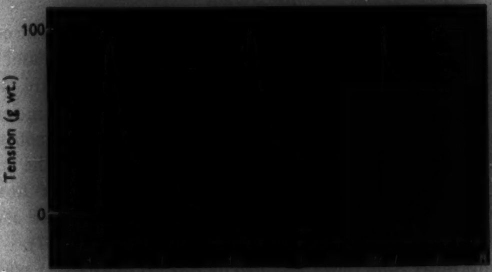

Fig. 3. Effect of inhibitory nerve stimulation on resting tension of the ABRM. d.c.=direct current stimulation, 3 V; *I*=stimulation of inhibitory nerve; *Str.*=stretch of muscle by 4 mm above reference length; *R*=release from stretch. Time marker 10 sec, temp. 14° C.

(4) Tonic contraction of the ABRM is accompanied by vigorous electrical activity due to discharges from muscle fibres (Hoyle & Lowy, 1956). In the stretched resting muscle no increase above the normal low level of sporadic spontaneous electrical activity is detectable.

(5) Maintained tension due to activity in the ABRM is associated with increased heat production (Abbott & Lowy, 1955). When the resting muscle is stretched, the only temperature change is a transient one which will be described in another paper.

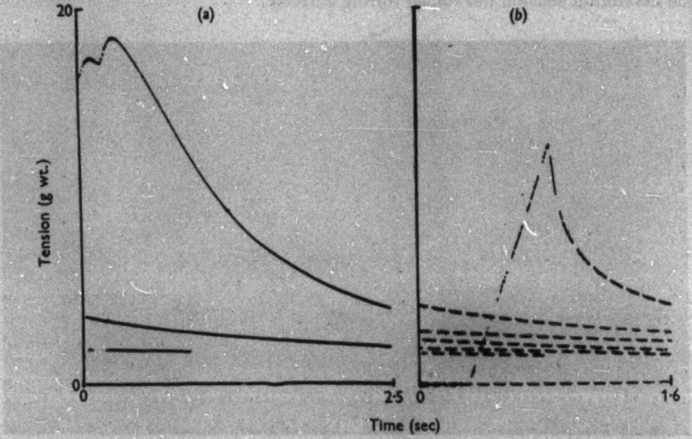

Fig. 4. *Helix* muscle. Records of tension decay (ordinates): *a*, following tetanic contraction at 29 mm; *b*, after stretch of resting muscle from 26 to 29 mm. Muscle weight 43 mg, temp. 14° C, reference length 30 mm.

Relaxation

In the *Helix* muscle, the fall of tension after an isometric tetanic contraction is practically exponential (Bozler, 1931; also record *a* of Fig. 4 and curve *a* in Fig. 5). The rate of relaxation increases with rise in temperature, as is shown in the middle curve of Fig. 6, where relaxation rate is plotted in terms of the rate constant per second of the exponential fall in tension. In contrast, stress relaxation follows a time course which can be described as the sum of two exponential components, one fast, the other slow (Abbott & Lowy, 1957). Changes in temperature have practically no effect on the rate constants of these two decay components (Fig. 6 and Abbott & Lowy, 1957).

These results differ from those obtained by Bozler (1930, 1931), who observed that stress relaxation follows the same single exponential time course as relaxation after isometric tetanic contraction, except when the muscle is stretched very rapidly—in that case the time course of stress relaxation showed an initial fast decay of tension followed by the usual slower curve. We have repeated and confirmed Bozler's results using his methods (recording on smoked drum, with snail blood as a bathing solution) (cf. Fig. 7). But we find that with improved techniques (transducer or optical lever and cathode-ray tube recording) the fast component of stress relaxation is present not only after rapid stretch but over a range of speeds of stretch from 1·5 to 20 mm/sec (cf. Fig. 3 in Abbott & Lowy, 1957). Furthermore, with the refined recording techniques, it is quite clear that the whole time course of stress relaxation always differs from the time course of relaxation after an isometric tetanic contraction (Fig. 4).

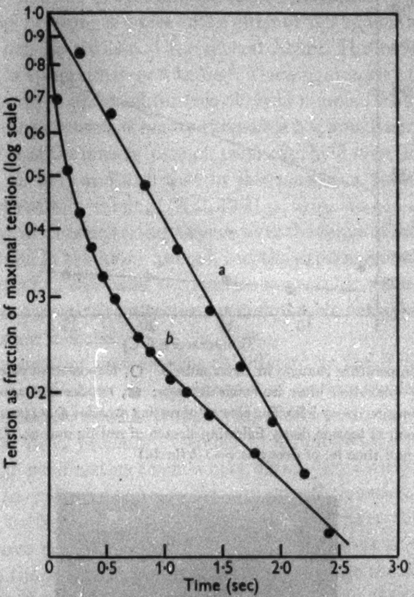

Fig. 5. *Helix* muscle. Same experiment as for Fig. 4. Curve (*a*), fall of tension in relaxation after isometric tetanus which produced a tension of 18·5 g; (*b*) decay of tension following stretch of resting muscle. Maximal tension produced, 12·6 g. Ordinates, tension as fraction of maximal value plotted on a logarithmic scale. (Curve (*a*) starts from the peak of contraction shown in Fig. 4*a*.)

This fact has also been demonstrated in many experiments with the *Mytilus* ABRM, with strip preparations from *Pinna's* posterior adductor and with the retractor of the squid's funnel. Even in inexcitable muscles initial decay of tension after the muscle has been stretched beyond reference length follows almost the same time course as in normal muscle.

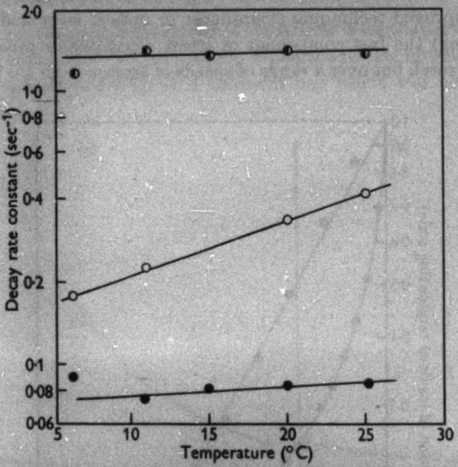

Fig. 6. Effect of temperature changes in *Helix* muscle. ○, Exponential rate constant of fall of tension during relaxation after isometric tetanus; ●, exponential rate constant of slow component of tension decay following stretch of resting muscle; ◐, exponential rate constant of fast component of tension decay following stretch of resting muscle. (Note: rate constant k is related to half-time (τ) of decay as $\tau = 1/k$ (ln 2).)

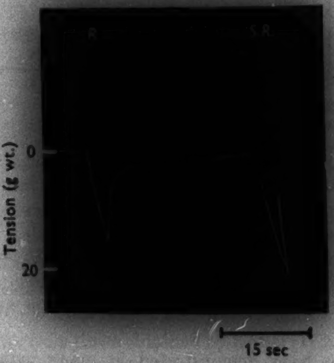

Fig. 7. Tension fall during relaxation (*R*) after tetanus, and during stress relaxation (*S.R.*). *Helix* muscle using smoked-drum recording. Temp. 14° C.

DISCUSSION

The long-fibred smooth muscles of *Helix* and *Mytilus* exert maximum active tension at about the longest lengths they have in the animal's body. In this respect their behaviour resembles that of vertebrate striated muscle.

The present experiments show that these molluscan muscles possess elastic properties when stretched beyond their maximum body length and that these elastic forces are not due to spontaneous tonic contraction. In the case of the *Helix* muscle, the breaking stress is of the order of 1–2 kg/cm^2 and a stress just less than that can be withstood for several hours. The breaking stress for *Mytilus* muscle is approximately 5 kg/cm^2. These figures are of the same order of magnitude as those obtained for frog skeletal muscle (Walter, 1947).

The fact that snail muscle is highly extensible has long been known and has led to the belief that the muscle 'cannot, passively, hold even the smallest load' (Bozler, 1936*b*, p. 261) and also that in this muscle 'a parallel elastic component is almost entirely missing' (Hill, 1950, p. 415). We can confirm Bozler's observation that there is no resting tension over the range of lengths which this muscle can assume in the living animal, but the experiments described in this paper show that when stretched beyond this range it can certainly hold a load which, reckoned per unit cross-section, is comparable with that which can be supported by other muscles.

It is rather surprising that muscles as different as the *Helix* retractor and frog sartorius should show the appearance of resting tension at about the length where isometric tension is maximal. Perhaps at this length the contractile material itself becomes extended to a limit where it resists further stretch, although such resting tension may decay very slowly. This applies to frog sartorius as well as to the molluscan smooth muscles. Thermo-elastic measurements on frog sartorius muscle (Hill, 1952) have shown that up to about 25% above reference length the tissue behaves as a rubber-like body with a positive thermo-elastic coefficient. In this range the resting tension is probably due to the contractile component (Hill, 1952). Above that length a thermo-elastic reversal occurs and the muscle shows a negative thermo-elastic coefficient similar to 'normal' elastic material, as the parallel elastic component begins to share the tension (Hill, 1952). In a similar way, the resting tension of snail muscle may in some part be due to the contractile component. It would be of interest to find out if in that muscle, with its supposed small parallel elastic component, the striking thermo-elastic reversal at high tensions appears at all.

The experiments described here on stress relaxation show that this process is not the same as the fall of tension after isometric contraction. The reason why this needs to be emphasized lies in Bozler's frequently repeated statement that in smooth muscle 'the decrease in tension after stretch follows the same time course as the isometric relaxation' (Bozler, 1941, p. 385, and 1953). This is so different from the corresponding events in striated muscle that, concerning this identity, Hill (1950, p. 416) asks 'can Bozler's results be true only at the particular temperature he used?' Our experiments show that the two processes are not identical within the temperature range used by Bozler.

The disagreement between the present results and those of Bozler is probably due to the fact that his 'smoked-drum' technique was unsuitable for the recording of small rapid changes in tension. Thus, in the case of the snail muscle, the early rapid changes in tension are attenuated. The resulting trace is smoothed out and gives a curve resembling closely that of relaxation after an isometric tetanic contraction (cf. Fig. 7).

We conclude, therefore, that there is no identity between the time courses of relaxation and of stress relaxation in smooth muscle any more than in striated muscle.

SUMMARY

1. Experiments with smooth muscle preparations from *Helix* and *Mytilus* have shown that maximum isometric tension is developed at about the longest length reached by the muscles in the animal's body (reference length).

2. When stretched beyond reference length the muscles exert resting tension. This is not due to spontaneous activity.

3. Relaxation, following isometric contraction, and stress relaxation (decay of tension following rapid stretch of resting muscle) are not identical processes as claimed by Bozler. Their time courses differ and stress relaxation is not significantly affected by changes in temperature.

We wish to thank the Director of the Plymouth Laboratory for his interest and encouragement. Part of the electrical apparatus was purchased with grants from the Royal Society.

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'BREATH-HOLDING' MEASUREMENT OF CARBON MONOXIDE DIFFUSING CAPACITY. COMPARISON OF A FIELD TEST WITH STEADY-STATE AND OTHER METHODS OF MEASUREMENT

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Previous papers from this laboratory (Carey, Phair, Shephard & Thomson, 1957; Shephard, Carey & Phair, 1958) have described a portable box-bag, the large version of which was apparently introduced originally by Roth (1939) and subsequently popularized by Donald & Christie (1949). This portable apparatus has already proved its worth in field survey measurements of carbon monoxide uptake, but, in view of current interest in the breath-holding technique of Krogh (1915) as modified recently by Forster and his colleagues (Forster, Fowler, Bates & Van Lingen, 1954; Forster, Cohn, Briscoe, Blakemore & Riley, 1955; Ogilvie, Forster, Blakemore & Morton, 1957), it was decided to investigate the potentialities of the breath-holding method as a portable field test. A special version of the portable box-bag with enlarged bellows and electric kymograph was developed for this purpose, and the opportunity was taken to make (1) a critical examination of certain variables affecting the breath-holding technique, and (2) a comparison of the consistency of breath-holding results with other methods, field and laboratory, for the measurement of pulmonary diffusing capacity.

METHODS

Portable breath-holding apparatus. A standard portable box-bag has been modified by the fitting of a larger bellows (4·5 l. capacity) and high-speed electric kymograph. The apparatus is still highly portable, weighing no more than 5·5 kg, although it has been necessary to increase the vertical height to about 80 cm. The technique is essentially that of Forster *et al.* (1954). The patient takes a few normal breaths from the outer compartment of the box. As soon as a steady respiratory level is reached he is asked to make a maximum expiration, and then inspires the test mixture (12 % He and 0·1 % CO in air) from the inspirate bag. After breath-holding, the subject makes a rapid, deep expiration, all but the first 750 ml. being collected in the expirate bag. After mixing with a 100 ml. glass syringe, samples are returned to the laboratory in special sampling bottles (Carey *et al.* 1957) for He and CO analysis. The standard formula (Ogilvie *et al.* 1957) is used to calculate the diffusing capacity. It should be noted that this formula requires a figure for the residual volume. In the present series this was determined by a standard closed-circuit technique, but in circumstances where this is not possible a normal value may be obtained from tables (Needham, Rogan & McDonald, 1954) or an approximate experimental value may be found by means of the box-bag (Carey *et al.* 1957).

Since the volume of gas collected in the expirate bag is rather small (1–2 l.) the opportunity for changes in gas composition due to diffusion is correspondingly increased. When 2 l. of the test mixture was placed in the original meteorological balloon of 30–40 l. total capacity, a considerable loss of both He and CO was observed in the space of a few minutes (Fig. 1). Such loss of gas is largely a surface-dependent phenomenon (Shephard, 1955), and in later models the problem has been minimized by the fitting of a much smaller bag (capacity *ca.* 10 l.) to collect expired gas.

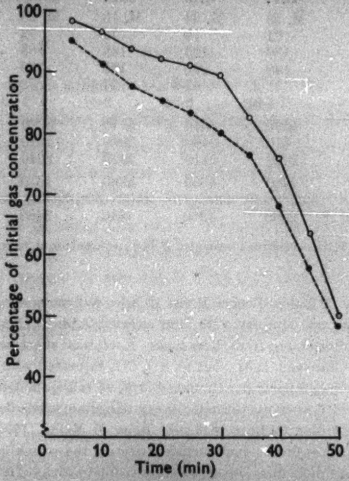

Fig. 1. Diffusional loss of helium, ●, and carbon monoxide, ○, from the original portable 'breath-holding' apparatus.

Portable box-bags. The original portable box-bag (Model I) has been described in detail elsewhere (Carey *et al.* 1957; Shephard *et al.* 1958). The subject breathes a mixture of 12% He and 0·1% CO for a period of 1 min, and analysis of inspirate and expirate bags allows the calculation of fractional CO uptake. In the modified apparatus (Model II), the subject first takes ten breaths of a 'supercharged' mixture containing 0·14% CO and 28% O_2 from the outer compartment of the box, and then proceeds as with Model I. End-tidal alveolar samples are collected at the beginning and end of the period of breathing from the bags. The CO uptake is then given by

$$(F_{I_{CO}} - F_{E_{CO}})\,V_I - (F_{A_{CO_t}} - F_{A_{CO_0}})\,V_F$$

where $F_{I_{CO}}$ and $F_{E_{CO}}$ are fractional concentrations of CO in inspirate and expirate, V_I is the volume of the inspirate mixture, $F_{A_{CO_0}}$ and $F_{A_{CO_t}}$ the fractional concentrations of CO in the end-tidal samples, and V_F the functional residual gas volume.

In calculating the diffusing capacity, it is important to remember the considerable change in alveolar CO concentration over the course of a respiratory cycle. The end-tidal concentration is lower than the mean value, and hence gives the 'highest likely value for diffusing capacity'

($D_{CO_{II}}$ of Bates, Boucot & Dormer, 1955). The 'lowest likely value' may be calculated from the end-tidal value and the known dilution of inspired gas mixture ($D_{CO_{III}}$ of Bates *et al.* 1955). The 'best value' for diffusing capacity is given by the mean of $D_{CO_{II}}$ and $D_{CO_{III}}$ ($D_{CO_{IV}}$). Finally, the alveolar CO concentration may be estimated by the Bohr equation, using an assumed value for physiological dead space ($D_{CO_{I}}$ of Bates *et al.* 1955).

Steady-state box-bag. This is essentially the circuit described by Bates *et al.* (1955). Fractional CO uptake and the several estimates of diffusing capacity may be calculated as with Portable box, Model II.

TABLE 1. Physical characteristics of subjects

	R.P.	R.L.	L.P.	R.J.S.	M.B.	M.V.
Sex, age (yr)	M. 23	M. 24	M. 19	M. 28	M. 35	F. 44
Height (in.)	71	76	74	72	69	66
(cm)	180	193	188	183	175	168
Weight (lb.)	160	215	170	160	166	161
(kg)	72·6	97·5	77·1	72·6	75·3	73·0
Body surface area (m^2)	1·91	2·28	2·01	1·93	1·89	1·81
Vital capacity (ml.)*	4590	5810	5710	5220	4270	3200
Exp. reserve (ml.)*	870	2450	2040	2210	830	810
R.V. predicted (ml.)*	2090	2110	2320	2240	1940	1760
F.R.C. Predicted (ml.)*	2960	4560	4360	4610	2770	2570
Box-bag I (ml.)*	3350 ± 110	4130 ± 70	4550 ± 90	3890 ± 70	2960 ± 150	3390 ± 40
Closed circuit (ml.)*	2980	4360	4810	3460	2190	3495

* ml. BTPS; R.V. = residual volume; F.R.C. = functional residual capacity.

Infra-red gas analyser. A Liston–Becker Model 15 infra-red gas analyser was used in all these experiments. The analyser was equipped with filter-cells containing CO_2 and water vapour, which eliminated much of the interference from these gases. A minimal effect persisted, due to broadening of the CO spectrum (Ramwell, 1957), but at 6 % CO_2 saturated with water vapour the error did not amount to more than 8 parts per thousand (1 % of full-scale deflexion). A more serious error was caused by rapid flow of gas through the sampling line, particularly during the collection of end-tidal samples. Gaensler, Cadigan, Ellicott, Jones & Marks (1957), commenting on this phenomenon, suggested it was due to dust particles entering the sampling chamber. The present author prefers to attribute difficulties during gas flow to fluctuations of temperature and pressure, since satisfactory readings were readily obtained by momentarily stopping the pump and allowing conditions in the sampling chamber to stabilize.

Subjects. Six healthy adults acted as subjects for these experiments. Physical details are given in Table 1. All were in the laboratory for a three-month period, and tests were performed in rotation, so that it was not necessary for any subject to undertake more than two experiments in any one day. Subjects rested for 5 min before testing, and no observations were made sooner than 1 hr after a heavy meal, but no attempt was made to secure truly basal conditions. All measurements were made with the subjects seated.

CO back pressure. Subject M.V. was virtually a chain smoker, but none of the other subjects took more than an occasional cigarette in the evening. The arterial CO was not determined, as it did not seem justifiable to submit each subject to 40–100 arterial punctures. As far as the breath-holding technique is concerned, it has been demonstrated clearly that the back-pressure of CO exerts quite a small effect on the results obtained (Forster *et al.* 1954). The back-pressure effect is also disregarded in most of the published 'steady-state' methods (Hatch, 1952; Filley, MacIntosh & Wright, 1954; Bates *et al.* 1955), although Forbes, Sargent & Roughton (1945) and more recently Linderholm (1957) have queried the validity of this assumption. The philosophy of the present author in this matter is simple. Provided the smoking habits of the patient are not disturbed, the back-pressure due to the resting arterial P_{CO} is relatively constant, and may be considered for practical purposes as an impairment of diffusing capacity. The additional back pressure created by breathing CO can be calculated using formulae developed by Linderholm (1957).

In a healthy subject, the CO tension rises by 0·002 mm Hg/min, giving an error of 1 % in the first minute and approaching 5 % at the fifth minute. The precision of available techniques does not warrant special corrections even in normal subjects. In patients where the CO uptake is pathologically reduced, the error from this cause will be even smaller. If the method is being used for diagnostic purposes, there may be some advantage in knowing the initial arterial P_{CO}, but the maximum reduction in apparent diffusing capacity at rest is no more than 10 % even in a heavy smoker, and this is unlikely to cause serious confusion of diagnosis. Indeed, if absolute values are desired, it would seem a simple matter to apply an empirical correction to the experimental data ranging from 2 % in the non-smoker to 10 % in a person smoking forty or more cigarettes per day.

RESULTS

Variables affecting the breath-holding technique

A full discussion of variables affecting the pulmonary diffusing capacity has recently been presented by Ogilvie *et al.* (1957), and it is only necessary to consider certain results that are not in agreement with their findings.

Adequacy of breath-holding. The two experienced subjects (R.J.S. and M.B.) found no problem in holding the chest without air movement for a 10-sec period of breath-holding. A third subject (R.P.) took a small additional inspiration (50 ml.) in one of ten tests. The remaining three subjects found breath-holding less easy. L.P. held his chest steady in four of ten experiments, but in the remainder continued to inspire 20–70 ml. of gas. Six out of ten records for subject R.L. were disturbed by repeated oscillations some 20 ml. in amplitude, while the performance of M.V. was even less satisfactory, five out of ten tracings showing oscillations of 50 ml. or more. However, there did not appear to be any relationship between the magnitude of these oscillations and experimental values for the pulmonary diffusing capacity.

Alveolar gas volume during test. Some 140 observations were made on two normal subjects over a wide range of alveolar gas volumes (Fig. 2). It can be seen that over the middle range of alveolar volumes (3·5–4·5 l.) the change of D_{CO} was small, amounting to 3·4 ml./mm Hg/min (19·2 %) in R.J.S., and 1·2 ml./mm Hg/min (7·0 %) in M.B. However, with deeper inspirations a considerable increase of D_{CO} occurred, and in R.J.S. the diffusing capacity at an alveolar volume of 6 l. was more than twice the value observed at a volume of 4 l.

Comparison with other methods. Ten observations were made on each subject by each of the methods detailed above. The data obtained are summarized in Table 2. In all cases the mean value for each method, the standard deviation of a single observation, and the coefficient of variation for a single observation have been calculated.

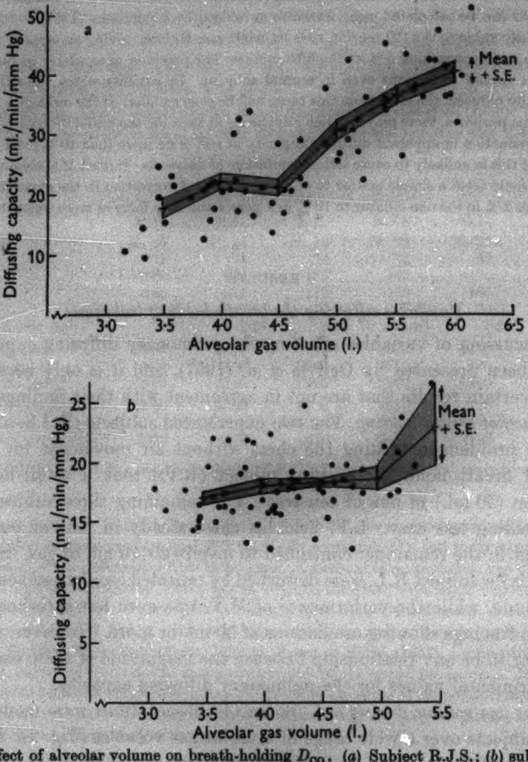

Fig. 2. Effect of alveolar volume on breath-holding D_{CO}. (*a*) Subject R.J.S.; (*b*) subject M.B.: ordinate scales differ.

DISCUSSION

The primary objective of devising a portable breath-holding apparatus for the field measurement of pulmonary diffusing capacity has clearly been achieved. Values for breath-holding D_{CO} obtained with this apparatus agree very well with previously published findings on normal subjects (Bates & Pearce, 1956; Ogilvie *et al.* 1957; Marks, Cugell, Cadigan & Gaensler, 1957). However, the original portable fractional uptake method has proved so successful in field use (Shephard *et al.* 1958) that the merits of rival methods need careful scrutiny. Perhaps the most obvious advantage of the breath-holding technique is that it estimates what was once considered a constant—the CO diffusing capacity.

TABLE 2. Comparison of different methods of measuring carbon monoxide uptake. Values for each subject represent mean of ten consecutive observations

	R.P.	R.L.	L.P.	R.J.S.	M.B.	M.V.	Mean for six subjects
Using portable single breath apparatus							
Mean D_{CO} (ml./min/mm Hg)	30·2	29·4	23·3	33·6	17·7	14·4	24·8
S.D. of single observation	2·62	2·22	3·91	6·30	2·49	3·15	3·45
Coefficient of variation (%)	8·7	7·6	16·8	18·7	14·0	21·9	14·6
Using portable box-bag, Model I							
(*a*) Fractional uptake							
Mean % uptake/min	48·6	28·0	31·6	30·9	32·6	20·9	32·1
S.D. of single observation	3·44	4·46	6·31	2·16	6·39	3·76	4·42
Coefficient of variation (%)	7·1	16·0	20·0	7·0	19·6	18·0	14·6
(*b*) Fractional uptake corrected to standard tidal volume of 890 ml.							
Mean % uptake/min	35·2	25·1	28·1	29·3	31·3	21·1	28·4
S.D. of single observation	2·52	3·57	5·22	2·29	6·39	3·76	3·96
Coefficient of variation (%)	7·2	14·2	18·6	7·8	20·4	17·8	14·3
Using portable box-bag, Model II							
(*a*) D_{CO} calculated from observed end-tidal gas samples (Method II of Bates *et al.* 1955)							
Mean D_{CO} (ml./min/mm Hg)	27·8	24·1	30·7	20·9	12·2	10·3	21·0
S.D. of single observation	5·53	4·17	11·10	2·21	2·06	2·29	4·56
Coefficient of variation (%)	20·0	17·3	36·1	10·6	16·8	22·1	20·5
(*b*) D_{CO} calculated from volume of gas inspired (Method III of Bates *et al.* 1955)							
Mean D_{CO} (ml./min/mm Hg)	20·3	20·4	24·3	16·8	10·9	9·2	17·0
S.D. of single observation	2·13	2·99	7·60	1·88	1·76	1·93	3·05
Coefficient of variation (%)	10·5	14·6	31·3	11·2	16·2	20·9	17·5
(*c*) Mean of (*a*) and (*b*) (Method IV of Bates *et al.* 1955)							
Mean D_{CO} (ml./min/mm Hg)	24·1	22·3	27·5	18·9	11·6	9·8	19·0
S.D. of single observation	3·68	3·56	9·22	2·00	1·92	2·10	3·75
Coefficient of variation (%)	15·2	16·0	33·6	10·6	16·4	21·4	18·9
(*d*) D_{CO} calculated by use of Bohr equation (Method I of Bates *et al.* 1955)							
Mean D_{CO} (ml./min/mm Hg)	17·0	19·1	19·1	17·4	11·0	8·2	15·3
S.D. of single observation	2·36	2·54	4·58	2·35	2·09	1·34	2·54
Coefficient of variation (%)	13·9	13·3	24·0	13·4	18·9	16·4	16·7
(*e*) Fractional uptake							
Mean % uptake	47·9	46·2	44·5	40·4	29·1	24·4	38·8
S.D. of single observation	5·71	4·41	4·46	3·29	2·81	3·22	3·98
Coefficient of variation (%)	11·9	9·5	10·0	8·2	9·7	13·2	10·4
Using 'steady-state' method							
(*a*) D_{CO} calculated from observed end-tidal gas samples (Method II of Bates *et al.* 1955)							
Mean D_{CO} (ml./min/mm Hg)	22·5	21·2	21·1	20·6	15·5	11·0	18·6
S.D. of single observation	4·61	3·56	4·05	3·62	2·20	1·38	3·24
Coefficient of variation (%)	20·6	16·8	19·2	17·6	14·2	12·6	16·8
(*b*) D_{CO} calculated from volume of gas inspired (Method III of Bates *et al.* 1955)							
Mean D_{CO} (ml./min/mm Hg)	19·3	17·6	15·7	17·9	13·5	9·6	15·6
S.D. of single observation	3·06	2·61	6·00	2·70	1·92	1·31	2·93
Coefficient of variation (%)	15·9	14·8	38·3	15·1	14·2	13·7	18·7
(*c*) Mean of (*a*) and (*b*) (Method IV of Bates *et al.* 1955)							
Mean D_{CO} (ml./min/mm Hg)	20·9	19·4	19·5	19·2	14·5	10·3	17·3
S.D. of single observation	3·88	2·98	3·48	3·01	2·05	1·32	2·79
Coefficient of variation (%)	18·6	15·4	17·8	15·7	14·2	12·8	15·8
(*d*) D_{CO} calculated by use of Bohr equation (Method I of Bates *et al.* 1955)							
Mean D_{CO} (ml./min/mm Hg)	23·0	18·2	19·6	20·2	14·2	9·1	17·4
S.D. of single observation	7·00	3·54	3·51	7·32	5·40	2·81	4·93
Coefficient of variation (%)	30·5	19·4	17·9	36·2	38·0	30·9	28·8
(*e*) Fractional uptake							
Mean % uptake	54·8	51·6	52·4	54·8	44·0	39·5	49·5
S.D. of single observation	1·26	1·25	1·12	1·72	2·34	7·13	2·47
Coefficient of variation (%)	2·30	2·43	2·15	3·14	5·31	18·1	5·57

While it is now well recognized that the diffusing capacity is not a constant, it still seems to many a less nebulous and variable quantity than the fractional CO uptake. Direct comparison of several methods of measuring diffusing capacity and fractional uptake in the present group of subjects suggests that the advantage is more apparent than real—indeed the simplest type of portable box-bag will give values for the fractional uptake (corrected to a standard tidal volume) that are if anything more reproducible than the breath-holding D_{CO} figures. It may have been noted that the coefficient of variation for breath-holding D_{CO} in the present series is appreciably larger than the value of 8·5% reported by Ogilvie *et al.* (1957) for a group of normal subjects followed over the course of several weeks. This may be due in part to the simpler recording system of the portable apparatus, but a more important factor is probably control of alveolar volume, which would be particularly difficult to standardize under field conditions. The present study suggests that alveolar gas volume has a more important effect on diffusing capacity than has been admitted by Ogilvie *et al.* (1957). Re-examination of the data on which their conclusions are based shows that even in the largest of their five subjects (height 1·88 m) the 'large' alveolar gas volume was not much over 5 l., while in one of the female subjects it was no more than 3·5 l. Observations made on subject R.J.S. suggest that if a really deep inspiration is taken the diffusing capacity can be doubled. A recent report from the Boston group (Marks *et al.* 1957) confirms the present author's findings and states categorically that changes of alveolar volume can produce 'differences of as much as 50% in the breath-holding D_{CO}'. The mechanism for this increase of D_{CO} may be transmission of a stretching force from the pulmonary tissues to the capillaries, increasing the available diffusing surface. This would probably be relatively unimportant over the normal range of alveolar volumes, but would become increasingly significant as the non-linear portion of the lung compliance curve was reached. The practical consequences of the variation in D_{CO} have been examined in the four subjects where the inspired volume was not deliberately varied. In different tests, the alveolar volumes still differed by as much as a litre (Table 3). If results for each subject shown in Table 3 are subdivided according to alveolar gas volume, differences of diffusing capacity become apparent even over this rather limited range, although the discrepancy is only large in subject M.V.

TABLE 3. Alveolar gas volumes in four subjects

Subject	R.P.	R.L.	L.P.	M.V.
Range of alveolar gas vol. (l.)	4·30–5·16	4·66–5·71	4·80–5·65	3·70–4·64
Small alveolar vol. (mean, l.)	4·45	5·11	5·11	3·79
Diffusing capacity (ml./min/mm Hg)	29·4	29·4	22·3	12·6
Larger alveolar vol. (mean, l.)	4·99	5·61	5·54	4·41
Diffusing capacity (ml./min/mm Hg)	30·8	29·1	24·2	16·2

It does not seem very practicable to correct the D_{CO} to a standard alveolar gas volume, since the shape of the curve relating the two variables differs in different subjects, and at least twenty observations would be required to establish the slope in any one person. An error of this order must therefore be accepted as a hazard of the breath-holding technique.

The fact that some subjects are unable to maintain an absolutely steady chest position for the 10-sec period of breath-holding does not appear to invalidate the results. This observation must be reconciled with present knowledge of gas-mixing in the respiratory structures of the lung. Although equilibrium is probably not reached as rapidly as was postulated by Rauwerda (1946), significant concentration differences in any one respiratory unit are certainly abolished within 2–3 sec (Shephard, 1957). Concentration differences between more widely separated parts of the lung may continue to diminish throughout the 10-sec breath-holding, but it seems unlikely that the rate of this process will be influenced greatly by the minor changes of alveolar gas volume (50–200 ml.) that commonly occur. It therefore seems reasonable to expect that unless changes in alveolar gas volume are large, or occur predominantly within the first 2–3 sec, acceptable values for the diffusing capacity will be obtained despite imperfect breath-holding.

In large-scale field tests, time and skill are also important factors in judging a method. The time required to complete the breath-holding test is no longer than with the original portable box-bag, but it does suffer the disadvantage of requiring a more skilled operator, since the results can be invalidated if the dead space is not washed out before the final gas sample is collected.

The absolute significance of the CO uptake as measured by the original portable box-bag (Model I) is limited, since expired gas is collected only for the first 50–70 sec. The results obtained are influenced by the rate of mixing of gas as well as by the CO uptake of the blood. Continuous records of expired CO concentration during the test have been obtained both in this laboratory and elsewhere (for example, Gilson & Hugh-Jones, 1955), and such records have shown that approximately ten breaths are necessary to produce a steady reading. The effect of the low initial alveolar CO concentration is to reduce the fractional uptake by about 25% during the first minute (Table 2). In pathological subjects with delayed gas-mixing the discrepancy would be greater, and in this respect it may be thought that the breath-holding technique is giving a purer measurement of the condition of the 'alveolar-capillary barrier'. However, neither the breath-holding technique nor the steady-state method is immune from effects attributable to inequalities of the ventilation: perfusion ratio, and indeed there would seem some danger that the breath-holding technique may over-estimate the diffusing capacity by allowing gas to diffuse into areas of the lung that are not accessible during the few brief seconds of a normal respiratory cycle.

The breath-holding technique may also be compared with the newer version of the portable box-bag (Model II). The latter apparatus gives very reproducible values for the fractional CO uptake and the results are no longer seriously affected by delays of gas mixing within the lungs, but attempts to determine the diffusing capacity with this apparatus have proved less successful. The difficulty seems related to the speed of the final expiration. Some subjects completed this rapidly, but in others the speed was variable, and absorption of CO during expiration gave low end-tidal gas concentrations. In four of the six subjects more reproducible results were obtained by application of the Bohr equation, using an assumed value for the physiological dead space. Bates *et al.* (1955) have pointed out that the Bohr equation gives very erroneous results for D_{CO} when the tidal volume is small. In some of their experiments tidal volumes averaged as little as 350 ml. However, with the portable box-bag technique the subject is required to maintain a stable and moderately deep tidal volume (800–1000 ml.), and under such conditions an assumed value for the dead space has relatively much greater accuracy. The present figures would suggest that for field survey work application of the Bohr equation could well replace attempts at collection of representative end-tidal samples. Calculations based on the amount of gas mixture inspired (Method III of Bates *et al.* 1955) also give rather variable results in some subjects. This is because the measured end-tidal gas composition again enters into the computation. Although it has not proved easy to obtain an accurate figure for the 'ideal' alveolar CO concentration by any of the above techniques, their use in normal subjects would seem justified. The results do not differ significantly from those obtained by the more involved blood-gas method of Filley *et al.* (1954), and the reproducibility seems somewhat better (S.D. 16–20%, as compared with a 'probable error' of 25%). It is possible the blood-gas method may give a more accurate picture of alveolar CO tension in pulmonary disease, but even this cannot be accepted without careful comparison of the two approaches.

The absolute magnitude of results from the breath-holding technique and the different types of portable box-bag must finally be compared with values obtained by the steady-state technique. Forster *et al.* (1955) stated that the breath-holding and steady-state methods gave similar results, but this conclusion was based on a rather limited number of observations, duplicates of which varied by as much as 400% (their Table 2, p. 1422). Subsequent reports have agreed that there is a difference, small in the results of Bates & Pearce (1956), larger in the work of Marks *et al.* (1957). In the present series the breath-holding D_{CO} is the greater in all subjects, the discrepancy being particularly marked in subject R.J.S. Marks *et al.* (1957) postulate that the breath-holding method is sampling the fastest ventilated and most rapidly perfused areas, but it is probably more true to say that, whatever part of the total pulmonary membrane is sampled, the long post-inspiratory pause of the breath-holding technique makes ventilation more effective in that area. The other and probably the more important factor is the alveolar gas volume, which is a litre or more larger in the breath-holding experiments.

The newer version of the portable box-bag (Model II) gives values for diffusing capacity that correspond fairly closely with those obtained by the steady-state method. On the other hand, the fractional uptake of CO is significantly greater in the steady-state method. This is undoubtedly related to differences in the tidal volume adopted during the two types of test. Perhaps the most interesting feature of the steady-state data is the small coefficient of variation with the fractional uptake method of calculation. Marks *et al.* (1957) point out, as did Bates (1952) in his original description of the method, that the results can be influenced markedly by hyperventilation; however, this is not a serious criticism if the pattern of ventilation is carefully controlled, as with the portable box-bag apparatus. The finding of a close correlation between ventilatory equivalent and fractional CO uptake (Marks *et al.* 1957) in no way invalidates the method; indeed, the existence of the correlation is hardly surprising since both quantities are dependent on diffusing capacity, alveolar gas tension, pulmonary capillary gas tension, ventilation, and a number of constants. When every possible criticism has been made, the fact remains that the standard deviation of the fractional uptake method is one-third of that encountered in the determination of diffusing capacity, and differences between different normal subjects also seem less marked. Despite its lack of sophistication, there would seem good grounds for continued use of the method, at least until sources of variation in the determination of D_{CO} are better controlled.

SUMMARY

1. An adaptation of the portable box-bag suitable for breath-holding measurements of carbon-monoxide diffusing capacity is described.

2. Repeated tests on six healthy normal subjects have shown that breath-holding D_{CO} varies in a non-linear manner with changes in total alveolar gas volume. During a deep inspiration D_{CO} may be doubled.

3. Some subjects find difficulty in holding breath steadily for 10 sec, but oscillations of up to 200 ml. in alveolar gas volume do not measurably alter the calculated D_{CO}.

4. Comparison of the portable breath-holding apparatus with the steady-state method and with other types of portable equipment has yielded D_{CO} values that show a very similar coefficient of variation, but absolute values are larger by the breath-holding technique, probably on account of the larger alveolar gas volume during the test.

5. Steady-state determinations of fractional carbon monoxide uptake have a smaller coefficient of variation than any available method for the calculation of D_{CO}, and this approach still seems the best where a simple field test of pulmonary diffusion is required.

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FACTORS INFLUENCING LIMB TEMPERATURE DURING EXPERIMENTS ON SKELETAL MUSCLE

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During a study of the influence of lowered body temperature on the action of neuromuscular blocking drugs, muscle temperature was recorded, as well as oral or rectal temperatures. It was observed that the temperature of the hind limbs was often lower than that of the rest of the body and also that the difference between limb and body temperature varied considerably from animal to animal. As changes in muscle temperature have a marked influence upon the action of neuromuscular blocking drugs it was decided to investigate factors contributing to the varying differences between limb and body temperature.

A preliminary account of this work has already appeared (Bigland & Zaimis, 1956).

METHODS

Cats, dogs and rabbits, the animals used in the present study, were anaesthetized with a mixture of chloralose (0·08 g/kg for cats, 0·11 g/kg for dogs and 0·1 g/kg for rabbits), and pentobarbitone sodium (3–6 mg/kg). The tibialis anterior muscle was prepared for recording, shielded silver electrodes were applied on the sciatic nerve and the nerve was ligated centrally to the electrodes. Twitches of the tibialis muscle were elicited by square-wave pulses of 0·2 msec duration and twice the strength required to evoke a maximal contraction. As little hair as possible was removed during dissection and all skin incisions were sewn up again to reduce heat loss. The hind limb was mounted on a Brown–Schuster myograph stand, and the tendon attached to a flat steel spring.

The apparatus for the recording of muscle temperature consists of a simple bridge circuit having a thermistor as one arm of the bridge. Any change in the thermistor resistance unbalances the bridge and the potential difference thus produced is fed into a four-stage d.c. amplifier. The amplified output is then fed into a driving unit consisting of a two-stage cathode follower amplifier and an electronic trigger circuit which operates a high-speed relay. This relay is connected to a unidirectional motor, mechanically coupled to a variable potentiometer, the latter forming another arm of the bridge circuit. The motor movements adjust the potentiometer to rebalance the bridge. A pointer, mechanically coupled to the unidirectional motor, records every change in the thermistor resistance on a smoked drum. The instrument is calibrated in degrees centigrade before use.

For the recording of muscle temperature the thermistor was inserted through a small incision in the skin so that its tip lay between the gastrocnemius and digitorum extensor longus muscles at about the middle of the limb. Once the thermistor was in position the skin was drawn up around it with a thread. Oral or rectal temperatures were measured by means of mercury thermometers.

RESULTS

Generally when the contractions of the hind-limb muscles of different mammalian species are recorded the limbs are immobilized in a horizontal or preferably a vertical position. In a first series of experiments, therefore, the influence of the position of the limb on muscle temperature was examined.

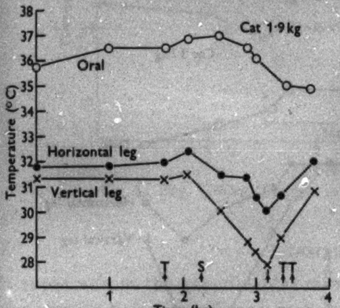

Fig. 1

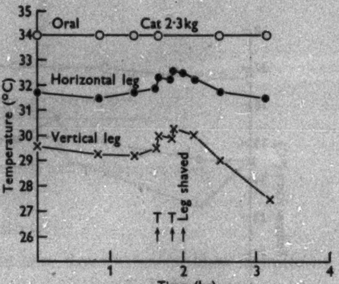

Fig. 2

Fig. 1. Cat, 1·9 kg. Changes in muscle and oral temperatures. In all experiments one limb was mounted horizontally and the other vertically; throughout each experiment both tibialis anterior muscles were stimulated indirectly with single shocks at 10 sec intervals, except where otherwise indicated. T, tetanus of 50 shocks/sec and 5 sec duration; S, stimulation stopped.

Fig. 2. Cat, 2·3 kg. Effect of shaving the limb on muscle temperature.

Both tibialis anterior muscles were prepared for recording and the limbs mounted, one vertically and the other horizontally. Blood pressure, muscle temperature and the contractions of both muscles, elicited indirectly at 10 sec intervals, were recorded simultaneously on a smoked drum. Oral or rectal temperatures were read at regular intervals.

In cats the temperature of both limbs was almost always lower than the oral or rectal temperature. The usual difference between the horizontal limb and body temperature was of 1½–2° C, but a difference of as much as 4° C was occasionally recorded. The vertical position, however, always increased the difference, in some experiments to as much as 5° C (Figs. 1–3). When stimulation of the muscles was discontinued the difference between body and limb temperature usually increased, but again the fall was more pronounced in the vertical limb (Fig. 1).

A reduction of muscle temperature could also be produced by shaving the limb. 'Shaving' here involved cutting off as much hair as could be conveniently removed with a pair of scissors, a procedure which is often used when preparing a limb for experiments on skeletal muscles. In the experiment illustrated in Fig. 2 the temperature of the vertical limb was 2° C lower than that of the horizontal one. This difference remained unaltered for 2 hr while the muscles were stimulated with single shocks at 10 sec intervals, or with tetani of 5 sec duration and 50/sec frequency. After both limbs had been shaved, however, the temperature of the vertical limb fell considerably more than that of the horizontal limb, thus increasing the difference in temperature between the two limbs.

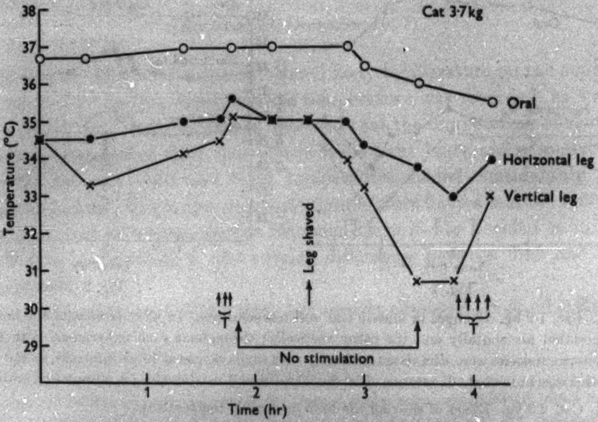

Fig. 3. Cat, 3·7 kg. Effect of shaving the limb and cessation of stimulation on muscle temperature.

In some animals the temperature of the limb was very little affected either by cessation of stimulation or by the 'shaving' process. A combination of the two, however, was always effective, particularly in the vertical leg. Fig. 3 shows the results of such an experiment. Cessation of stimulation had little effect but when this was followed by 'shaving' the temperature fell by 2° C in the horizontal limb and by 4° C in the vertical one. This figure also shows that after a short series of tetani the temperature of the limb was increased.

The difference between limb and body temperature in dogs was small in comparison with that in cats, and in some instances non-existent. When present this difference was seldom affected by shaving or cessation of stimulation (Fig. 4).

Rabbits occupied an intermediate position between cats and dogs. There was usually less difference between limb and oral temperatures than in cats, but muscle temperature fell sharply after fur had been removed, especially in the vertical leg.

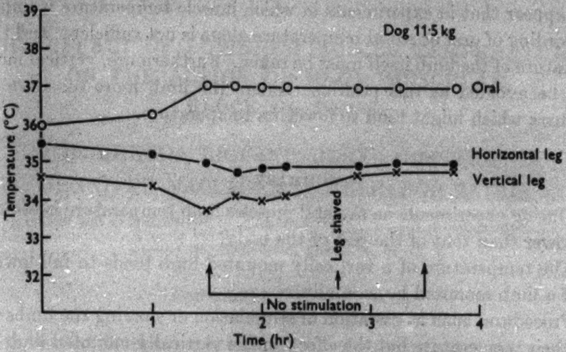

Fig. 4. Dog, 11·5 kg. Changes in muscle and oral temperatures.

DISCUSSION

It appears from the experimental results here demonstrated that in spite of every precaution taken during preparation of the animal, limb temperature, especially in cats, is lower than that of the body. Moreover, muscle temperature is considerably affected by the position in which the limb is mounted. The temperature of a limb in the vertical position tends to fall lower than that of a limb mounted horizontally. Cats, again, proved to be the most sensitive of the three species under consideration, dogs the least and rabbits somewhere between the two. Furthermore, any procedures such as cessation of stimulation or shaving the limb, which tend to lower muscle temperature, have a more pronounced effect in the vertically mounted limb. Dogs have proved to be less sensitive to these procedures than cats and rabbits. This lesser sensitivity of dogs is probably due to a more efficient blood flow through their limbs and a small dependence on hair for insulation, at least in the types of short-haired dogs used in the experiments.

It was not possible to correlate the size of the animal or its initial blood pressure with the degree of difference between limb and body temperatures. Rabbits are generally smaller animals than cats but their limb temperature proved less easily affected. Furthermore, great differences between body and muscle temperatures were found in large as well as small cats. Moreover, a much lower limb than body temperature was sometimes found in animals with high initial blood pressures.

The fact that experimental conditions can affect limb temperature is important, for temperature changes have a marked influence on drugs active at the neuromuscular junction (Bigland, Goetzee, Maclagan & Zaimis, 1958). On this account and because of the results obtained in the present study, it would appear that in experiments in which muscle temperature is important, the recording of oral or rectal temperature alone is not sufficient, and that the temperature of the limb itself must be taken. Furthermore, vertical mounting should be avoided as this position renders the limb more sensitive to any procedures which might tend to lower its temperature.

SUMMARY

1. During experiments on skeletal muscles limb temperature is often found to be lower than that of the rest of the body.
2. The temperature of a vertically mounted limb tends to fall lower than that of a limb mounted horizontally.
3. Procedures such as cessation of stimulation or shaving the limbs tend to lower their temperature but the effect on the vertically mounted limb is more pronounced.
4. Of the three mammalian species studied, the limb temperature of the cat proved to be the most easily affected, that of the dog the least and that of the rabbit somewhere between the two.
5. It is suggested that in experiments on skeletal muscles, in which temperature is important, vertical mounting should be avoided and the temperature of the limb itself should be recorded.

We wish to express our thanks to the Medical Research Council for a grant (to B. B.) and a grant for technical assistance (to E. Z.). We are indebted to Mr P. Whittington for designing and constructing the apparatus for the recording of muscle temperature, and to Mr R. Owen for his technical help.

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THE EFFECT OF LOWERED MUSCLE TEMPERATURE ON THE ACTION OF NEUROMUSCULAR BLOCKING DRUGS

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One of the recent developments in surgery is the performance of operations on patients whose body temperature has been lowered, but little is known of how this procedure affects the action of drugs given during operation. Holmes, Jenden & Taylor (1951), in experiments carried out on the isolated diaphragm of the rat, have demonstrated that cooling reduces the effect of tubocurarine, a substance which interrupts neuromuscular transmission by competition with acetylcholine. In view of this it was felt it might be of importance to find out how the action of depolarizing neuromuscular blocking drugs, such as suxamethonium and decamethonium, is modified by changes in skeletal muscle temperature.

METHODS

Cats and dogs were anaesthetized with a mixture of chloralose (0·08 g/kg for cats, and 0·11 g/kg for dogs) and pentobarbitone sodium (3–6 mg/kg) injected into the subcutaneous vein of the forelimb or the internal saphenous vein. The dogs also received 2 mg/kg of morphine hydrochloride subcutaneously 30–60 min before they were anaesthetized. Hind limbs were set up horizontally on a Brown–Schuster myograph stand, shielded silver electrodes were placed on the sciatic nerve, and the nerve ligated central to the electrodes. Twitches of the tibialis muscle were elicited by square-wave pulses of 0·2 msec duration, and twice the strength required to evoke a maximal twitch. The muscle was attached to a flat steel spring, and its contractions recorded on a smoked drum.

Body temperature was varied at the rate of 4° C/hr by passing hot or cold water through a thin rubber bag inserted into the abdominal cavity or by modifying the external heating. This method made it possible to produce a slow but steady fall in body temperature without perceptible shivering. In some experiments, while body temperature was kept normal the temperature of one limb was lowered by the external application of rubber bags containing crushed ice. Oral or rectal temperatures were measured by means of mercury thermometers and muscle temperature by the use of a thermistor (Bigland & Zaimis, 1958).

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Experiments *in vitro* were performed on the isolated phrenic–diaphragm preparation of the rat (Bülbring, 1946) with the modifications introduced by Goffart & Ritchie (1952). For the electrical recording of the twitch tension an RCA 5734 mechano-electronic transducer valve was used.

RESULTS

In cats, even when all precautions are taken during preparation, muscle temperature is often below oral or rectal temperature. In some experiments this difference was found to be as much as 4° C. Further, muscle temperature is easily lowered by local factors, such as the position of the limb, the presence or absence of stimulation, or the amount of fur covering the limb (Bigland & Zaimis, 1956). For these reasons, in the present experiments the limb was fixed in a horizontal position, oral or rectal temperatures were measured at regular intervals and muscle temperature was continuously recorded. As the circulatory system is usually dangerously affected at low body temperatures, the present study was carried out on animals whose body temperature was never brought below 27° C.

Substances blocking by long-lasting depolarization

Tubocurarine interrupts neuromuscular transmission in all mammals by competition with acetylcholine. The mode of action of decamethonium and suxamethonium on the other hand is not uniform. In some mammalian species, such as the cat, these compounds block by mimicking acetylcholine (Zaimis, 1951, 1954; Paton & Zaimis, 1952; Bovet & Bovet-Nitti, 1955). Burns & Paton (1951) demonstrated that the likeness of decamethonium to acetylcholine rests on the ability of the compound to cause a persistent depolarization of the end-plate region. In other mammalian species, however, decamethonium and suxamethonium exhibit, initially, an acetylcholine-like action, but during the blocking process their action changes into that of a substance competing with acetylcholine. When first observed this mode of action was described as 'dual' (Zaimis, 1953). For these reasons, in the present study cats only were used for the experiments with suxamethonium and decamethonium.

The tibialis anterior muscle of the cat shows no significant change in sensitivity to successive doses of suxamethonium when given at regular time intervals and at normal body temperature; the same muscle exhibits a slowly decreasing sensitivity to repeated doses of decamethonium (Jewell & Zaimis, 1954). When, however, the neuromuscular blocking effects of successive doses of both drugs were tested at different muscle temperatures, it was found that the block produced by the same dose was greater both in magnitude and duration the lower the temperature. Fig. 1 illustrates the results obtained in an experiment in which suxamethonium was used. The first dose, administered when the muscle temperature was 38° C, produced the usual short-lasting interruption of neuromuscular transmission. As the muscle temperature fell the duration of the blockade became more and more prolonged. The effect was completely reversible on rewarming the animal. Fig. 2 illustrates similar results with decamethonium. By measuring the 50% recovery time it was found that with decamethonium and suxamethonium a change in temperature from approximately 36 to 31° C caused a fourfold increase. This effect was completely reversed on rewarming.

Fig. 1. Records of maximal twitches of the tibialis anterior muscle of a 2·8 kg cat in response to indirect stimulation delivered once/10 sec. At the arrows (↑) intravenous injections of 150 μg suxamethonium diiodide.

Fig. 2. Records of maximal twitches of the tibialis anterior muscle of a 4·1 kg cat in response to indirect stimulation delivered once/10 sec. At the arrows (↑) intravenous injections of 120 μg decamethonium diiodide.

In mammals a lowering of muscle temperature is accompanied by a decrease in the maximal twitch tension. This reduction of the contraction (at least over a temperature range of 36–26° C) is not the result of an effect of cooling on neuromuscular transmission, for the twitch shows the same changes after denervation (Maclagan & Zaimis, 1957). Figs. 1 and 2 illustrate this effect. When, however, single doses of a neuromuscular blocking drug are administered at different muscle temperatures, such a reduction of the twitch tension by cooling does not complicate the interpretation of the results obtained, as the magnitude of the block is always measured in relation to the size of the twitch just before the administration of the drug.

In a second series of experiments suxamethonium was administered in the form of a continuous slow infusion. In some animals the infusion was started at normal body temperature. Fig. 3 shows the result of an experiment of this type in which the rate of infusion was adjusted to produce a block of about 25% and was then kept constant while body temperature was first lowered and then restored to normal. It can be seen that the magnitude of the neuromuscular block followed the temperature variations faithfully. In order to eliminate the complicating factor of the reduction of twitch tension produced by cooling, further experiments were performed in which the infusion of suxamethonium was started after the body temperature had been lowered. Fig. 4 illustrates the results of such an experiment. After body temperature was lowered to 29° C infusion was started and the rate adjusted to produce a 100% block. Then while the rate was kept constant the animal was rewarmed. Again, the magnitude of the block followed the temperature variations.

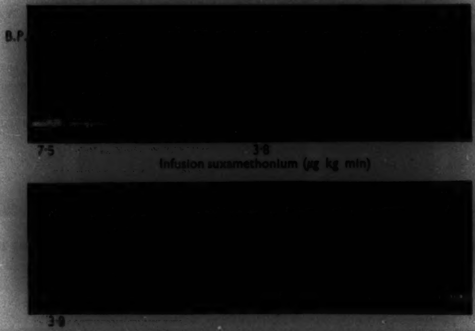

Fig. 3. Records of arterial blood pressure and of maximal twitches of the tibialis anterior muscle of a 3·3 kg cat in response to indirect stimulation delivered once/10 sec. Effect of changes in body temperature during intravenous infusion of suxamethonium diiodide.

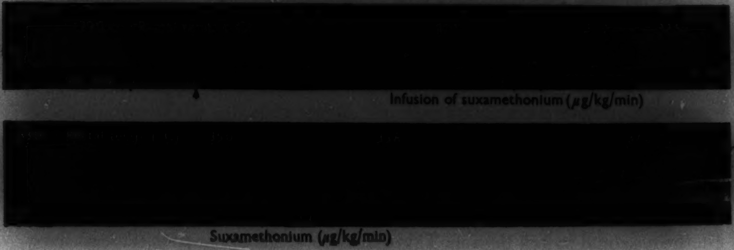

Fig. 4. Records of maximal twitches of the tibialis anterior muscle of a 2·3 kg cat in response to indirect stimulation delivered once/10 sec. Effect of raising body temperature during intravenous infusion of suxamethonium diiodide. Before infusion the body temperature had been lowered to 29° C. (For details see text.)

Although cooling considerably prolongs the action of the depolarizing drugs, the nature of the blockade is in no way affected however long the paralysis lasts. A tetanus is always well sustained and does not antagonize the block, neostigmine is ineffective or prolongs the block and tubocurarine antagonizes it.

In experiments on the isolated diaphragm of the rat the action of suxamethonium was studied at various bath temperatures. The results were similar to those obtained in the whole animal. Cooling greatly increased both the magnitude and the duration of the blockade.

Substances blocking by competition with acetylcholine

Holmes *et al.* (1951) have demonstrated on the isolated diaphragm of the rat that tubocurarine is less effective at lowered muscle temperature, but it was felt that additional information from experiments *in vivo* was needed. As tubocurarine is only slowly destroyed and excreted (Marsh, 1952), and successive doses produce cumulative effects, even at normal body temperature, the analysis of the results obtained *in vivo* is rather difficult. In order to minimize this difficulty alternative ways of cooling the muscles and of administering the drug were devised.

Fig. 5. Records of maximal twitches of the tibialis anterior muscle of a 5·2 kg dog in response to indirect stimulation delivered once/10 sec. At the arrows (↑) intravenous injections of 0·5 mg tubocurarine chloride at different muscle temperatures as indicated on top of the records.

In a first series of experiments the effect of successive small doses of tubocurarine at different muscle temperatures was studied. With doses producing less than a 40% reduction of the maximal twitch tension, the effect was always smaller at the lower temperatures. On rewarming the animal the effect increased again. Fig. 5 illustrates such an experiment and shows also that even with small doses cumulative effects can be seen. Although 2 hr had elapsed between the first and second doses of tubocurarine, both administered at normal muscle temperature, the second dose produced a greater effect.

In a further series of experiments the body temperature and the temperature of one leg were kept normal, while the temperature of the other leg was lowered by external cooling. Thus, a temperature difference of about 5–6° C between the two limbs was maintained throughout the experiment. Fig. 6 illustrates the results of an experiment in which two doses of tubocurarine were administered, one preceding and the other following a dose of decamethonium. In this figure the tracings, which have been aligned vertically, are simultaneous records of contractions of the tibialis anterior muscles of both legs. Cooling of the muscle potentiated the action of decamethonium, while it reduced that of tubocurarine. Fig. 7 shows the results obtained with another animal in which the cold leg was rewarmed at the end of the experiment. Again, when there was a temperature difference between the two limbs the cold muscle was less affected by tubocurarine. Tubocurarine, however, produced the same degree of block in both tibialis muscles after the cold leg had been rewarmed.

Fig. 6. Records of maximal twitches of the tibialis anterior muscle of the right and left limbs of a 2·8 kg cat in response to indirect stimulation delivered once/10 sec. The body temperature was 37° C but the temperature of the left hind limb was maintained at 30° C throughout the experiment by local application of external cooling. The arrows (↑) refer to intravenous injections of 1 mg tubocurarine chloride (at 1 and 3) and of 200 μg decamethonium diiodide (at 2).

Fig. 7. Records of maximal twitches of the tibialis anterior muscle of the right and left limbs of a 2·6 kg cat in response to indirect stimulation delivered once/10 sec. The arrows (↑) refer to intravenous injections of tubocurarine chloride (0·5 mg at 1 and 3; 1 mg at 2). The body temperature was 37° C throughout the experiment; the temperature of the right hind limb was kept at 31° C by local application of external cooling during the 1st and 2nd, but rewarmed between the 2nd and 3rd injection.

In another series of experiments the temperature of the whole animal was lowered with the exception of one leg which was kept warm by the application of external heating, and tubocurarine was administered in the form of a slow continuous infusion. Fig. 8 shows the results of such an experiment, in which the right leg was kept warm by the application of external heating. Part *A* illustrates the contractions of both tibialis muscles at different temperatures before the infusion of tubocurarine was started. Part *B* shows the steady state of block in both muscles reached 45 min after the start of the infusion. Between *B* and *C* the body temperature was brought back to normal but the temperatures of the limbs were maintained unchanged by the application of external cooling to the left leg. Rewarming of the body did not alter the degree of block.

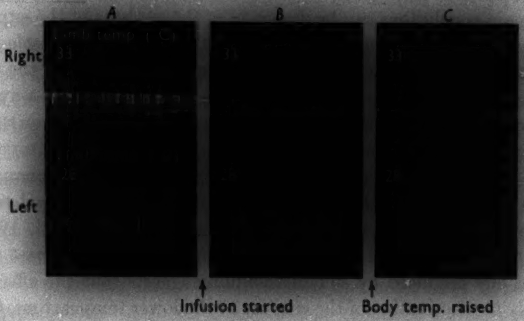

Fig. 8. Records of maximal twitches of the tibialis anterior muscle of the right and left limbs of a 2·8 kg cat in response to indirect stimulation delivered once/10 sec. Effect of intravenous infusion of tubocurarine chloride (5·6 mg/kg/min) at different limb and body temperatures. Body temperature at *A* and *B* lowered to 33·5° C and brought back to normal at *C*. The temperature of the limbs indicated on top of the records. *A* before, *B* and *C* during infusion; *B*, 45 min after the start of infusion; *C*, some time later after rewarming the animal. (For details see text.)

Two conclusions may be drawn from these experiments, (*a*) that cooling reduces the magnitude of a blockade produced by tubocurarine but does not affect its duration, and (*b*) that the local temperature of the limb is the determining factor. The only experiments in which the blockade produced by tubocurarine was prolonged at lowered muscle temperatures were those in which successive large doses of the drug were administered. It seems that the temperature effects were here masked by the cumulative effects of the drug. This conclusion is supported by the fact that on rewarming the muscle the prolongation of the effect is only slightly curtailed. The slow excretion and destruction of tubocurarine, however, is not the only factor which complicates such experiments. A large dose of tubocurarine often produces a profound and long-lasting fall in blood pressure which is further increased by cooling. Thus, in addition to the slow excretion and destruction of the substance an impaired peripheral circulation may complicate the picture.

DISCUSSION

It is clear from the experimental results presented above that changes in muscle temperature greatly affect the action of neuromuscular blocking drugs.

Three main conclusions can be drawn from the analysis of the experiments with suxamethonium and decamethonium: (*a*) that lowered muscle temperature greatly increases both the magnitude and the duration of the action of depolarizing drugs, (*b*) that the nature of the blockade is in no way affected however long the paralysis lasts, and (*c*) that the effect is reversed on rewarming the muscle. Decamethonium is a substance which is not destroyed in the body and is almost quantitatively excreted in the urine in unchanged form (Zaimis, 1950). On the other hand, suxamethonium is rapidly hydrolysed, mainly by plasma pseudo-cholinesterase (Bovet & Bovet-Nitti, 1955). The fact that a block produced by decamethonium is more, or at least as much, affected as one produced by suxamethonium suggests that the effect cannot be due only to inactivation of the enzyme at the lower temperature, for if this were so the block produced by suxamethonium should be the most affected. This suggests that cooling influences the process by which a long-lasting depolarization of the motor end-plate interrupts neuromuscular transmission. Such a suggestion is supported by the findings of Csapo & Wilkie (1956) who, studying the effect of potassium on frogs' skeletal muscles, found that at low temperatures the recovery from the depolarization produced by potassium is very slow and that a brief period of rewarming leads to a sudden and dramatic recovery.

Analysing the results obtained with tubocurarine the following conclusions can be reached: (*a*) that in experiments in which the cumulative effects of the drug are minimized, cooling reduces the magnitude of the blockade but has little influence on its duration, and (*b*) that on rewarming the muscle this effect is reversed. A lowering of muscle temperature will undoubtedly have the same influence on the action of acetylcholine as on that of suxamethonium and decamethonium. Thus, any increase in the effectiveness of acetylcholine by cooling will automatically reduce the action of any drug trying to compete with it. That the reduced activity of tubocurarine is due to an increased effectiveness of acetylcholine at low temperatures is also suggested by the observation that cooling reduces only the magnitude of the tubocurarine block, leaving the duration unaltered. The ability of a given dose of tubocurarine to produce a large or a small effect depends on the ease with which the drug can compete with acetylcholine. The duration of the blockade, however, is governed by factors, such as the rate of excretion and destruction of tubocurarine, which determine how long and in what concentration this drug is present at the neuromuscular junction.

A lowering of body temperature is usually accompanied by bradycardia and a fall in blood pressure. Such effects will tend to slow the blood flow through skeletal muscles. However, the experimental results suggest that the changes in the action of neuromuscular blocking drugs occurring at lower muscle temperatures are independent of changes in blood flow. Such a conclusion is reached because (*a*) a lowering of muscle temperature affects the action of depolarizing and competitive neuromuscular blocking drugs in opposite directions; (*b*) in isolated preparations, where there is no blood supply, the effect of lowered muscle temperature is just the same—increased activity of depolarizing drugs and reduced activity of tubocurarine; (*c*) the results obtained have always been dependent on local muscle temperature—for example, the same results have been obtained in animals whose muscle temperature changes have been produced by altering body temperature, or by altering the temperature of one limb only; and (*d*) the increased or decreased activity of neuromuscular blocking drugs at lowered muscle temperature is independent of the effect of cooling on the blood pressure and heart rate. In some experiments, particularly those on dogs, cooling produced no change in blood pressure and heart rate but had the same marked effect on the action of neuromuscular blocking drugs. The only effect which can be attributed to changes in blood flow is the more gradual onset of the blockade in the cold muscle, an effect seen with both depolarizing and competitive neuromuscular blocking drugs.

In man suxamethonium interrupts neuromuscular transmission by mimicking acetylcholine. Because of its brief action the drug is often administered during operations in the form of a slow continuous infusion. When the infusion is stopped neuromuscular transmission usually recovers rapidly. From time to time, however, anaesthetists come across cases in which suxamethonium produces a prolonged apnoea. Much work has been done, and many theories formed, in attempts to explain this phenomenon, but so far no satisfactory reason for it has been found. If this prolonged apnoea is due to a prolonged neuromuscular blockade of the respiratory muscles, then the cause of it may well be a fall in the temperature of these muscles.

SUMMARY

1. The influence of muscle temperature on the action of neuromuscular blocking drugs was studied. Cats and dogs were used for the experiments *in vivo*, and the isolated rat diaphragm preparation for the experiments *in vitro*.

2. Lowered muscle temperature increases the magnitude of the effect of depolarizing drugs and markedly prolongs their duration of action. On re-warming, these effects are reversed. The nature of the blockade is in no way affected however long the paralysis lasts.

3. In contrast, cooling reduces the magnitude of a block produced by tubocurarine, a substance which interrupts neuromuscular transmission by competition with acetylcholine.

4. The results suggest that cooling influences the process by which a long-lasting depolarization of the motor end-plate interrupts neuromuscular transmission.

5. It is suggested that a lowering of muscle temperature may be an important factor in cases where suxamethonium, a depolarizing drug, produces prolonged apnoea in man.

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THE EXCITATION OF RENSHAW CELLS BY PHARMACOLOGICAL AGENTS APPLIED ELECTROPHORETICALLY

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It has been reported that a group of cells lying within the ventral horn of the lumbar segments of the spinal cord of the cat are normally discharged by the release of acetylcholine from the terminals of the motor axon collaterals (Eccles, Fatt & Koketsu, 1954; Eccles, Eccles & Fatt, 1956). These cells, the Renshaw cells, in turn inhibit adjacent motoneurones. Some of the pharmacological properties of these pathways have already been investigated (Curtis, Eccles & Eccles, 1957). The anomalous behaviour of these cells when tested with certain drugs, specifically acting on peripheral cholinergic synapses, given either intravenously or intra-arterially, has suggested that they are surrounded by at least two diffusional barriers. In order to investigate these barriers more closely, methods have been devised by which drugs can be applied directly from a micro-electrode in the vicinity of the cell. These methods have been used in an investigation of the neuromuscular junction (del Castillo & Katz, 1955; 1957*a–c*).

METHODS

All experiments have been performed upon Renshaw cells located in the lower lumbar segments of the spinal cord of the cat under light pentobarbital anaesthesia. Since these cells are often difficult to locate, it was important to be able to test any one cell with a variety of pharmacological agents, and therefore five-barrelled electrodes were used. Four barrels, each containing a different drug in solution, specified in Table 1, were arranged radially around a central barrel containing 4M-NaCl, which was employed as the electrode recording the responses of the cell. Renshaw cells were located by finding areas within which the responses of a single cell to impulses in motor axon collaterals could be recorded. These impulses were set up by stimulating the cut ventral root of the appropriate segment (i.e. orthodromic or synaptic stimulation of the Renshaw cell). The composite electrode was held in a micromanipulator (Eccles, Fatt, Landgren & Winsbury, 1954), and once located the responses of a single cell could be recorded extracellularly for 3–4 hr. The spikes recorded were within the range 1·5–0·4 mV.

It was possible to apply positive or negative voltages either constantly or in pulses to any of the four drug-containing barrels. The voltage on each barrel was monitored by means of a cathode follower and the current flowing through the barrels could be recorded either by a galvanometer or on one beam of the oscilloscope after amplification. The current-carrying lines were connected via resistances of 100 MΩ (cf. Coombs, Eccles & Fatt, 1955) and chlorided silver wires to the barrels containing the drugs. A small negative backing voltage of 200–300 mV was applied to each barrel to prevent diffusion of the active cation from the tips (del Castillo & Katz, 1955).

Care had to be taken in these experiments that the electrical resistance of any one barrel was not too high. The recording barrel was connected to another cathode follower by a chlorided silver wire and the total input capacity of the system was at least 30 pF. Consequently, the electrode resistance had to be kept low (between 4 and 10 MΩ) in order to record the rapid spike potentials of Renshaw cells without considerable distortion. However, it was more important to have drug-containing barrels of low resistance. Not only does the resistance of micro-electrodes often increase considerably while passing through the spinal cord, but also resistance changes occur during the passage of current through them (Coombs, Curtis & Eccles, unpublished observations). Both these effects are more marked with electrodes of high resistance and consequently the resistance of the drug-containing barrels usually was kept below 100 MΩ. It was of special importance that the resistance of the barrel containing acetylcholine be kept constant because, as will be seen in the next paper (Curtis & Eccles, 1958), the usual testing procedure for blocking and potentiating drugs was to apply a constant voltage to the acetylcholine electrode for 3–4 sec at different testing intervals. Only if the resistance was constant could the same dose of acetylcholine be delivered on these different occasions.

TABLE 1. Drugs used, approximate concentrations and molecular weights

	Concn. (M)	mol. wt. (approx.)
Acetylcholine bromide	1–2	226
Nicotine hydrochloride	7–10	235
Carbaminocholine chloride	0·5	182·7
Acetyl-β-methylcholine	3	195·7
Arecholine hydrobromide	0·25	236
Succinylcholine bromide	0·25	155
Tubocurarine chloride	0·04	785·7
Procaine hydrochloride	1	272·8
Dihydro-β-erythroidine bromide	0·5	250
Decamethonium iodide	0·14	512
Prostigmine bromide	0·2	303·2
Edrophonium chloride (Tensilon, Roche Products Ltd.)	0·16	246

The electrodes were first filled by boiling in distilled water, which was then extracted by suction with a fine Polythene tube and replaced from above with the drug solution. At least 48 hr were allowed to elapse before use. This method was preferred to that of del Castillo & Katz (1957*a*), which depended on the prior use of 3M-KCl solution, because measurements of the resistance of each barrel gave an indication as to when the drug was uniformly distributed in it. The tips of the multibarrel electrodes were broken off at the required diameter (1–2 μ) with a fine glass rod during microscopic observation. Most drugs were used in nearly saturated solution. The approximate concentrations and molecular weights are shown in Table 1. Electrodes containing succinylcholine were stored at 5° C, thus reducing the rate of break-down of this substance (Fraser, 1954).

Although it is possible to give the total quantity of electricity used in passing quantities of drugs from the tips of electrodes (cf. del Castillo & Katz, 1957*a*), these values are only of use when comparing the responses of a single unit to drugs passed from one electrode. The distance from the electrode tip to any one cell was indeterminate and varied from cell to cell. It was certainly much greater than the distance between the orifices of individual barrels. Since the coupling resistances between any two barrels (cf. Coombs *et al.* 1955) were of the order of 5–10 KΩ and as the currents passed rarely exceeded 50×10^{-9} A, the interaction between barrels was negligible.

RESULTS

Random activity and synaptic stimulation

When the appropriate ventral root was stimulated antidromically, the normal type of response was obtained from Renshaw cells. The initial frequency of 700–1000/sec decreased over the first 10–50 msec (Fig. 1*A*, *C*), the actual duration of the discharge varying considerably from cell to cell. By using repetitive stimuli 10–20 msec apart it was possible to drive a single Renshaw cell to discharge at frequencies of 800–1000/sec for many seconds. This observation, together with the fall in frequency observed with a single stimulus, suggests that the observed response to synaptic stimulation is due to the fall in concentration of a synaptic transmitter which is initially released in a large amount, rather than to the cell becoming insensitive to the transmitter.

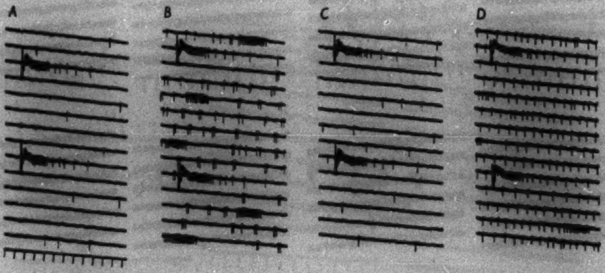

Fig. 1. The responses of Figs. 1, 2, 3*D–F* and 5 were recorded on moving film at such a rate that each sweep occupied approximately 130 msec and the delay between sweeps was less than 5 msec. *A*, *C*, random responses together with discharges evoked by maximal synaptic stimuli. The individual high-frequency responses are not clearly apparent owing to the slow sweep speed used. *B*, as for *A* and *C*, but simultaneously a current of 5×10^{-8} A was passing through a barrel containing acetylcholine. *D*, as for *B* but a current of 10×10^{-8} A flowed through a barrel containing nicotine. In both *B* and *D* the drug-evoked frequency was maximal. Time marker, 10 msec.

Most Renshaw cells discharge spontaneously and several types of random response occur. Single spikes are illustrated in Fig. 1*A*, *C*. Some cells discharged in units of two or three (Fig. 2*A*) and occasionally bursts of impulses occurred having a frequency and duration not unlike that obtained by synaptic stimulation (Fig. 1*B*). This phenomenon was more apparent during the application of acetylcholine (Fig. 1*B*) and nicotine (Fig. 1*D*). All types of spontaneous or background discharge were suppressed for some time after a response to a synaptic stimulus. In Fig. 2*A*, *D* the spontaneous spikes are absent for 0·3–0·5 sec after such a response and also in Fig. 1*A*, *C*, where the background discharge is lower. When the discharge rate was increased, either as in Fig. 1*B* by acetylcholine or as in Fig. 1*D* by nicotine, this depression was also apparent but was of shorter duration, especially when the background rate was high (Fig. 1*D*).

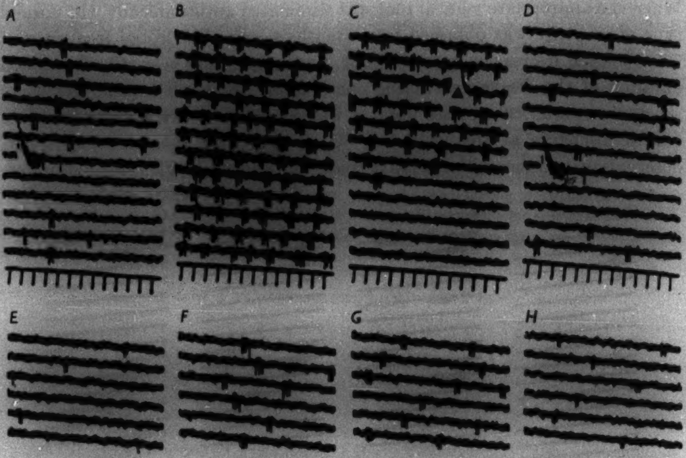

Fig. 2. All responses are from the one cell. *A*, background discharges before and after one synaptic stimulus. *B*, *C*, *D*, continuous records; in *B* and *C* (up to ▲) 3×10^{-8} A passed through the acetylcholine barrel; one synaptic stimulus in *D*. *E*, background discharge. *F*, recorded whilst 3×10^{-8} A flowed through a barrel containing acetyl-β-methylcholine. *G*, 4 sec after this current ceased. *H*, 30 sec after *G*.

Acetylcholine

In contrast to the variable responses found when acetylcholine is administered intra-arterially (Eccles *et al.* 1956; Curtis *et al.* 1957), all Renshaw cells have responded to acetylcholine which was released locally, either by removing the backing voltage or by applying a positive voltage to the electrode. When a cell was found, the backing voltage was usually reduced until it just prevented emission of acetylcholine by diffusion, as gauged by the effect on the cell. This was important, especially when repetitive short pulses of acetylcholine were used, because the magnitude of the backing voltage has a considerable effect upon the rate at which acetylcholine leaves the tip of the electrode. No accurate estimate could be made of the distance between the tip of the electrode and the cell because, in order to avoid damaging the cell, once reasonable discharges were obtained in response to synaptic stimulation and to acetylcholine the electrode was not moved.

The duration of the application of acetylcholine could be measured accurately, but because of the latency of the response, brief pulses (less than

0·1 sec) were rarely used. Usually the current was applied by a manually operated switch for 3–9 sec. In those cells responding with short latency, brief pulses of acetylcholine were released, and in several instances the minimal doses for single responses of individual cells was approximately 10^{-14} equiv of acetylcholine (del Castillo & Katz, 1955). The minimum latency varied considerably over a range 30–1000 msec and was least for any one cell when large doses were used. Most cells responded with repetitive discharges of single impulses (Fig. 3*A*, *B*) but with some the impulses were grouped in doubles or even in triples (Figs. 1*B*, 2*B*, 3*D*). During the continued application of a constant amount of current the frequency of discharge of any one cell diminished with time. The graph of Fig. 4*A* shows that, when a constant current of 10^{-8} A was passed through an electrode containing acetylcholine, the maximum frequency of 49/sec was attained in 1–2 sec and the frequency fell to 18/sec in 1 min. Consequently, when testing the effect of different current strengths or of potentiating or blocking drugs on an individual cell, it was necessary to apply acetylcholine for periods of only 3–4 sec. In addition, interference between doses was avoided by allowing an interval of at least 15 sec between tests. Under these circumstances a maximum frequency was obtained which depended on the rate at which acetylcholine was applied. In Fig. 4*B* are shown two strength-frequency curves obtained at different times from the one cell. In each case the indicated current was passed for 4 sec and

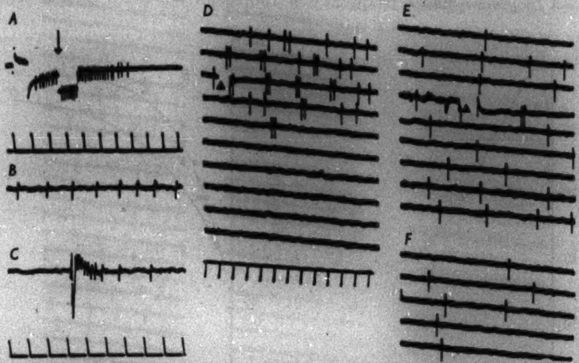

Fig. 3. *A*, discharge evoked by a current of 10×10^{-9} A flowing for 90 msec through a barrel containing acetylcholine; the step on the sweep to the right of the arrow has been expanded in *B*. *C*, the same sweep speed as *B* showing the responses evoked by a maximal synaptic stimulus. Time marker, 100 msec for *A*; 10 msec for *B* and *C*. *D*, responses produced 3 sec after the commencement of 15×10^{-9} A through the acetylcholine barrel; current ceased at ▲. *E*, responses produced by 100×10^{-9} A through the succinylcholine barrel; current ceased at ▲. *F*, 10 sec after *E*. Time marker, 10 msec.

the maximum frequency measured. Similar curves were drawn for several cells and in all cases increase in the rate of application of acetylcholine above a critical value did not give any further increase in frequency. However, this maximum was always less than that obtained when nicotine was used to fire the cell (cf. Fig. 1 *B*, *D*), which in turn was less than the maximum frequency obtained when the cell was stimulated synaptically. In Fig. 3 *A* a pulse of acetylcholine was used to stimulate a Renshaw cell and the step in the sweep, to the right of the arrow, is expanded in Fig. 3 *B*. The frequency of 100/sec was the maximum for this cell even when larger doses of acetylcholine were applied for a longer time. However, with synaptic stimulation (Fig. 3 *C*) the initial six responses had a frequency of 700/sec.

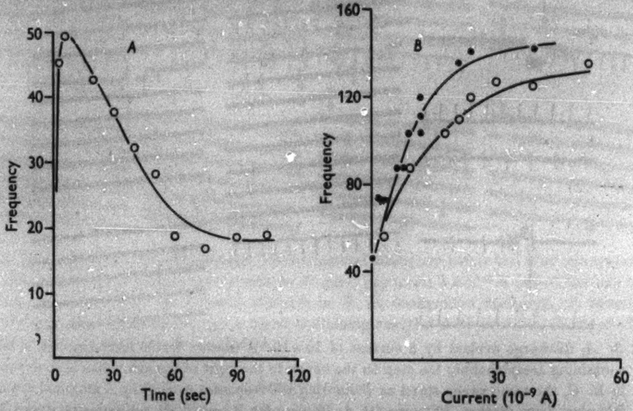

Fig. 4. *A*, frequency of discharge of a Renshaw cell evoked by a constant current of $1{\cdot}25 \times 10^{-8}$ A flowing through a barrel containing acetylcholine, plotted as a function of time. *B*, each point represents the maximum frequency of discharge of a Renshaw cell produced by the indicated current flowing for 3–4 sec through an acetylcholine electrode, there being 15 sec interval between tests. The two series ●, ○ are 10 min apart.

When the acetylcholine current ceased, the cell usually stopped responding within 0·5 sec (Figs. 2 *C*, 3 *A*, *D*, 5 *A*), although several cells showed discharges for 2–3 sec. If a background discharge was initially present, it reappeared after a quiescence of 1–2 sec.

Substances with an acetylcholine-like action

When applied by intra-arterial injection, nicotine stimulates Renshaw cells more effectively than does acetylcholine, whereas acetyl-β-methylcholine, carbaminocholine, arecholine and succinylcholine are without effect (Eccles *et al.* 1956). Nicotine when locally applied was also extremely effective in discharging Renshaw cells. Although the latency usually exceeded that found for acetylcholine, as illustrated in Figs. 5*B* and 6, the maximum frequency of discharge exceeded that obtained using acetylcholine sometimes by a factor of two (cf. Fig. 1*B*, *D*). Further, as also shown in Figs. 5 and 6, when the current through the nicotine-containing barrel ceased to flow, the discharge rate often

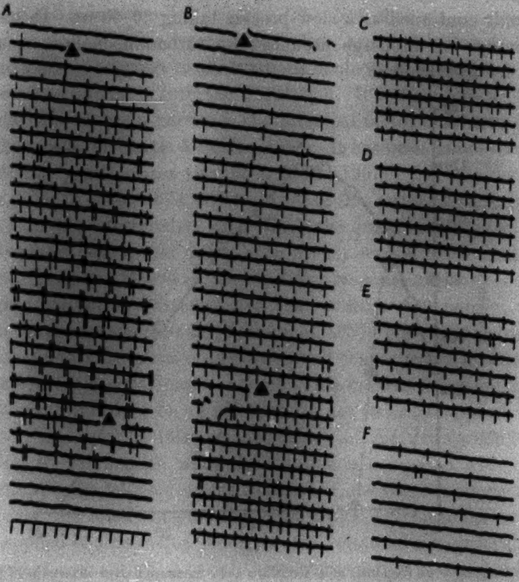

Fig. 5. All records are from the one cell that had almost no background discharge. In *A* a current of 4×10^{-8} A flowed through the acetylcholine barrel between the two ▲ points. In *B*, taken 5 min later, the same current flowed through the barrel containing nicotine for a time indicated by the two ▲ points. *C*, *D*, *E* and *F*—5, 10, 15 and 20 sec, respectively, after the second ▲ in *B*. Time marker, 10 msec.

continued to rise to a maximum and then gradually fell over a period of at least 1 min. On this account an extensive investigation into the dose–response relationships could not be carried out; nevertheless, it was clear that the maximum frequency of discharge evoked by nicotine always was less than that produced by synaptic stimulation of the cell.

The other stable acetylcholine-like substances were not very effective when applied in this manner. Succinylcholine produced a low-frequency discharge (Fig. 3*E*), which persisted for minutes after the backing voltage had been restored to the electrode. The responses of Fig. 3*F* were taken 15 sec after this time, whereas the discharge produced by acetylcholine (Fig. 3*D*) persisted for less than 1 sec when this drug ceased to flow. Similar results were obtained with acetyl-β-methylcholine (Fig. 2*E*–*H*), carbaminocholine and arecholine. In most cases the latency of the onset of any effect exceeded that found with acetylcholine, the maximum frequency of response was lower than that of acetylcholine and the cessation of firing when current had ceased to flow from the electrode continued as a slow process taking 30–60 sec. In one cell the maximum rate of discharge produced by carbaminocholine equalled that produced by acetylcholine.

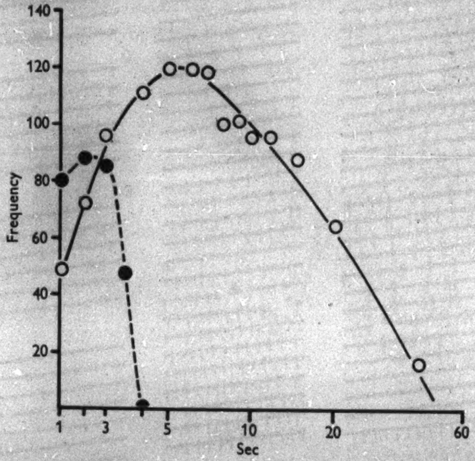

Fig. 6. The frequency of response of a Renshaw cell, measured over intervals of 0·5 sec, and evoked by acetylcholine, ●, and nicotine, ○; semi-log scale. The electrophoretic currents began at zero and ceased at 3 sec. Full series illustrated in Fig. 5.

DISCUSSION

The difficulties and limitations associated with the electrophoretic application of drugs from glass micro-electrodes have been discussed fully by del Castillo & Katz (1955, 1957*a*). However, a particular difficulty in this present investigation concerns the complete absence not only of visual control of the relationship between the electrode tip and the cell, but also of the ability to orientate the electrode easily with respect to a cell. Although it is possible to make a series of parallel tracks within the spinal cord in the region of a single cell and thereby to improve the recording of its responses, this method is time-consuming and often results in destruction or loss of the unit. Accordingly, once a cell was obtained which responded to stimulation both synaptically and by acetylcholine, further pharmacological investigation was undertaken. In most cases the extracellular responses were less than 1 mV and it is probable that the cells were 10–40 μ from the electrode, which offers an explanation of the long latencies found for the actions of some drugs.

The main results of this investigation are concerned with the relative effectiveness with which acetylcholine, nicotine and related substances depolarize a Renshaw cell when applied locally. The fact that all cells tested have responded to acetylcholine, carbaminocholine, succinylcholine and acetyl-β-methylcholine is in contrast to observations made when these drugs have been administered by injection into the circulation. It is therefore probable that the so-called blood–brain barrier is the principal factor obstructing access to the Renshaw cells (cf. Curtis & Eccles, 1958). It has been demonstrated at the neuromuscular junction (del Castillo & Katz, 1957*b*) that the post-synaptic receptors differ in their sensitivity to drugs of this series. Hence, it would be expected that the effectiveness with which the drugs investigated could discharge Renshaw cells would differ, even if they were applied directly to the post-synaptic region. However, the repetitive response of these cells produced by synaptic activation has suggested that a diffusional barrier, intimately related to the synaptic terminals (Eccles *et al.* 1956), prevents the free diffusion of acetylcholine from its site of liberation and consequently maintains a high concentration for a considerable period. This barrier, functioning to retain acetylcholine, would also be an effective barrier to drugs applied externally. This factor explains the relative inefficiency of the depolarizing agents in discharging Renshaw cells, when their action is compared with the rate at which synaptically released acetylcholine can discharge a single unit. It is unlikely that the low-frequency discharge which acetylcholine evokes when directly applied is a consequence of the activation of a very localized group of post-synaptic receptors. Not only have comparatively large doses of acetylcholine been discharged from the electrode, compared with those used at the neuromuscular junction (del Castillo & Katz, 1957*a*), but the cell size is probably small in comparison to the distance from which the drug is released. Consequently, the actual concentration of acetylcholine would be expected to be virtually uniform around the cell boundaries. By limiting the rate at which electrophoretically applied acetylcholine reaches the post-synaptic receptors, this barrier could also account for the shape of the dose–response curve of Fig. 4*B*.

The relatively short duration of response occurring when acetylcholine ceased to flow from the electrode is presumably due to the local action of cholinesterase both external to and internal to the synaptic barrier. As will be seen in the subsequent paper (Curtis & Eccles, 1958), under the influence of a locally applied anti-cholinesterase the responses evoked by acetylcholine and by synaptic stimulation are greatly increased and prolonged. The responses evoked by nicotine and the other acetylcholine-like substances are not complicated by enzymatic removal and the time course probably parallels the removal of the drug purely by diffusion. The occurrence of random discharges of Renshaw cells both in single and multiple groups can be ascribed to the leakage of quanta of acetylcholine from the presynaptic terminals, as has been observed at neuromuscular junctions (Fatt & Katz, 1952). This spontaneous discharge can be recorded with single electrodes containing 3 M-KCl or 4 M-NaCl, hence it is not a spurious result due to undetected diffusion from an electrode containing a depolarizing agent. Further, the discharges are not a result of cellular injury, for not only can they be detected at constant frequency by a stationary electrode for several hours, but the frequency often remains unaltered in spite of moving the electrode tip 200–300 μ. The bursts of activity, not unlike that seen with synaptic stimulation, are possibly due to impulses in motor axon collaterals as a consequence of the discharge of the associated motoneurone. However, it is interesting that this type of activity is more common when there is a background of acetylcholine or nicotine, for it is unlikely that it is due to the discharge of motoneurones produced by these drugs. Since very little convergence is necessary to fire Renshaw cells (Eccles *et al.* 1954) the possibility exists that these trains of repetitive responses arise from an axon reflex. It has been demonstrated that a recurrent discharge occurs from muscle when the motor nerve is stimulated (Masland & Wigton, 1940; Eccles, Katz & Kuffler, 1942; Lloyd, 1942) due presumably to the action of released acetylcholine upon the nerve terminals. Consequently, if applied acetylcholine initiates impulses in the terminal branches of the motor-axon collaterals, these impulses, spreading to the associated endings of the same collateral, might give rise to repetitive discharges of a Renshaw cell not unlike those evoked by stimulating the ventral root.

The phenomena of the depression observed in the background discharge following either the response to synaptic stimulation or to applied acetylcholine is probably related to the finding that acetylcholine can desensitize the post-synaptic receptors of muscle (Thesleff, 1955; Katz & Thesleff, 1957). Thus, following a period of repetitive activity due to a relatively high concentration of acetylcholine, the actual sensitivity of the post-synaptic receptors to acetylcholine might become slightly decreased. This may also account for the 'accommodation' observed in Fig. 4*A*, where, under the influence of a constant rate of application of acetylcholine, the frequency of the discharge of the cell diminished with time.

SUMMARY

1. Methods are described by which drugs can be applied electrophoretically to Renshaw cells located within the ventral horn of lumbar segments of the cat's spinal cord.

2. These cells discharge spontaneously at low frequency. The rate of discharge is increased by acetylcholine, nicotine and related compounds having actions at peripheral cholinergic junctions.

3. There is evidence for the existence of two diffusional boundaries between these cells and substances administered intravenously or intra-arterially.

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THE EFFECT OF DIFFUSIONAL BARRIERS UPON THE PHARMACOLOGY OF CELLS WITHIN THE CENTRAL NERVOUS SYSTEM

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For many years it has been realized that the presence of the 'blood–brain barrier' has hindered the access of systematically administered drugs to possible central sites of action. The finding that Renshaw cells within the spinal cord are sensitive to acetylcholine and related substances, has provided a unique situation for the testing of drugs having known actions at peripheral cholinergic junctional regions. However, it seems likely that these cells are protected from some circulating drugs by at least two diffusional barriers. For example, the action of the blood–brain barrier is indicated by the finding that prostigmine is a very effective anticholinesterase when injected locally, but is almost ineffective when given intravenously (Eccles, Eccles & Fatt, 1956; D. R. Curtis, unpublished observation). The existence of a second barrier (cf. Eccles, Fatt & Koketsu, 1954; Curtis & Eccles, 1958), more intimately related to the synaptic terminals upon Renshaw cells, is suggested not only by the duration of the repetitive discharge produced by a single volley in the motor axons, but also by the relative ineffectiveness of the action of some drugs upon the first two or three responses.

The development of methods by which drugs can be applied to the immediate locality of Renshaw cells (Curtis & Eccles, 1958) overcomes the problem of the blood–brain barrier and allows an investigation to be made not only of the barriers associated with Renshaw cells, but also of the pharmacological properties of these cells.

METHODS

The methods have been described in the previous paper (Curtis & Eccles, 1958). In several early experiments, because of the relative ineffectiveness of tubocurarine chloride (DTC) applied electrophoretically, the investigation was repeated after the electrodes had been tested on a neuromuscular preparation. A saturated solution of DTC, being approximately 0·04 M, is an extremely poor conductor and in most cases the electrode resistance exceeded 300 MΩ. With the available polarizing currents the maximum current obtainable was 25×10^{-9} A and the possibility existed that DTC was not being passed out of the electrode in sufficient amount. However, with much lower currents sufficient DTC could be electrophoretically injected upon the end-plate region of an isolated rat-diaphragm preparation to block the effect of acetylcholine applied electrophoretically from an adjacent barrel.

RESULTS

Anticholinesterases

When administered intravenously, prostigmine has very little action upon the cholinesterase associated with Renshaw cells (Eccles *et al.* 1956). However, when applied electrophoretically, prostigmine behaves as a typical anticholinesterase, having an action on Renshaw cells similar to that produced by eserine when administered intravenously. In Fig. 1 *A* are shown three records of the response of a single Renshaw cell to a volley evoked in the ventral root of that segment. Following this, a current of 2×10^{-8}A was passed through the barrel containing prostigmine bromide and the upper, middle and lower records of Fig. 1 *B* and of Fig. 1 *C* were taken 55, 60, 75 and 90, 92, 94 sec later, the backing voltage being restored to the prostigmine barrel after the records of Fig. 1 *C* were taken. The enormous prolongation of the responses is obvious, particularly in the lowest record of Fig. 1 *C*. This effect of prostigmine had a latency of 45–60 sec but persisted for a much longer time. The records of Fig. 1 *D* were taken from the same Renshaw cell 17 min after those of Fig. 1 *C*.

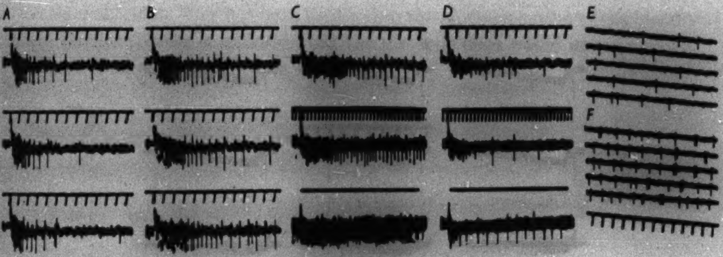

Fig. 1. *A–D*, records showing discharge evoked by synaptic stimulation of a single Renshaw cell. *A*, three control responses. A current of 2×10^{-8}A was then passed through the prostigmine-containing barrel for 90 sec: the three responses of *B* were recorded 55, 60 and 75 sec after the commencement of this current, those of *C* at 2 sec intervals following its cessation and those of *D* 17 min later; time marker, 10 msec for *A*, *B* and upper two records of *C* and *D*, 1 sec for the remaining records of *C* and *D*. *E*, discharge produced by acetylcholine: *F* as for *E* but a current was passing prostigmine from another barrel of the electrode, having commenced 1 min previously; time marker, 10 msec.

Not only did prostigmine increase the rate of discharge of those cells firing spontaneously, but it also increased the rate of response of the cell to applied acetylcholine. The records of Fig. 1 *E* and *F* were both evoked by a current of 7×10^{-8}A flowing through a barrel containing acetylcholine, but for a minute before *F* a current of 2×10^{-8} A had been flowing through the prostigmine barrel as well, with the consequence that the acetylcholine has become much more effective. Further, but not illustrated here, prostigmine prolonged for many minutes the discharge evoked by injected acetylcholine. In the absence of prostigmine the responses usually ceased within 1 sec of the cessation of the application of acetylcholine (cf. Curtis & Eccles, 1958).

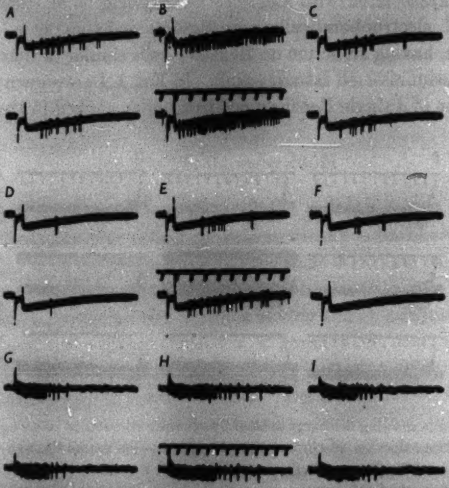

Fig. 2. Discharges evoked from a single Renshaw cell. *A–C*, current pulse of 12×10^{-9} A flowing for 50 msec through an acetylcholine electrode. *D–F*, as for *A–C* but for a current 4×10^{-9} A. *G–I*, synaptic stimulation by a volley in the segmental ventral root. Two successive records are illustrated for each type of stimulus. After the control records *A*, *D* and *G*, edrophonium was ejected from another barrel of the electrode by a current of 10^{-7} A; *B*, *E* and *H* were recorded during this; *C*, *F* and *I*, 20 sec after the edrophonium current ceased; time marker, 10 msec.

Similar results were obtained with edrophonium (Tensilon: 3-hydroxyphenyldimethylethyl-ammonium chloride, Roche Products Ltd.), which is structurally related to prostigmine and has a similar anticholinesterase activity (Randall, 1950; Nastuk & Alexander, 1954; Katz & Thesleff, 1957). When administered intravenously this drug has no effect on Renshaw cells. In Fig. 2*A* and *D* acetylcholine has been ejected from the electrode by the use of electrical pulses, the pulses being just threshold in *D*. The control response to synaptic stimulation by a volley in the ventral root is shown in Fig. 2*G*. A current of 10^{-7} A was passed through the electrode containing edrophonium, and 20 sec later the responses shown in Fig. 2*B*, *E* and *H* were recorded, using the same stimuli respectively as for *A*, *D* and *G*. After these records the edrophonium current was terminated and 1 min later the responses *C*, *F* and *I* were recorded again with the appropriate stimuli.

Thus edrophonium and prostigmine behave as reversible anticholinesterases, accentuating the action of acetylcholine, whether released synaptically or injected electrophoretically. Neither edrophonium nor prostigmine prolonged the response of a cell to nicotine or carbaminocholine.

Blocking drugs

Tubocurarine (DTC) is a powerful inhibitor of acetylcholine action at the neuromuscular junction (del Castillo & Katz, 1957*a*), but is virtually without effect upon Renshaw cells when administered intravenously or intra-arterially (Eccles *et al.* 1956). This has been ascribed to the blocking action of the blood-brain barrier (Eccles *et al.* 1956), but the methods used would not permit an accurate localization of the site of the barrier.

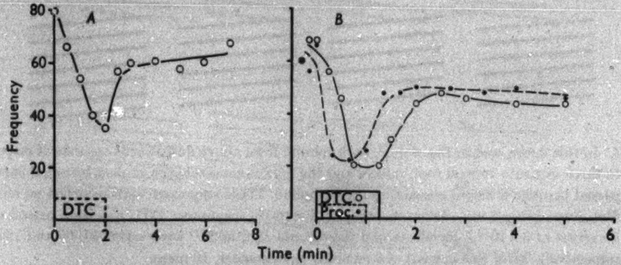

Fig. 3. *A*, maximum frequency of discharge of a Renshaw cell evoked at various intervals by equal currents passed through an acetylcholine-containing barrel for periods of 4 sec. From zero to 2 min DTC was passed from another barrel of the electrode using a current of 6×10^{-9} A. *B*, similar curves showing the effect of procaine (•) passed for 1 min (Series of Fig. 5 *A–F*) compared with that of tubocurarine (○) which was passed for 1·25 min; the current in both cases was $1{\cdot}5 \times 10^{-8}$ A. Abscissa scales differ.

When applied locally DTC was usually a relatively ineffective depressant on the responses both to synaptic stimulation and to electrophoretically injected acetylcholine. This effect was often so small that all the electrodes containing DTC were tested upon a rat-diaphragm preparation (see Methods). It was found that in spite of the high resistance of the barrels, a considerable quantity of DTC was always being passed ionophoretically.

On several occasions, however, DTC had a considerable effect upon the discharge of a Renshaw cell, evoked by acetylcholine applied from a nearby electrode. In the series illustrated in Fig. 3 *A* tests were made at the indicated times by passing a current for 4 sec through the acetylcholine barrel of the electrode. The same current was used on each occasion, control observations having indicated that under these circumstances, and provided that tests were made at intervals exceeding 15 sec, the maximum frequency of discharge of the cell attained in individual tests varied by less than 10%. A current of 6×10^{-9} A was used to pass DTC from the electrode for 2 min. The acetylcholine response was diminished. On cessation of the DTC injection, there was an initial rapid rate of recovery, but recovery was still incomplete 5 min later.

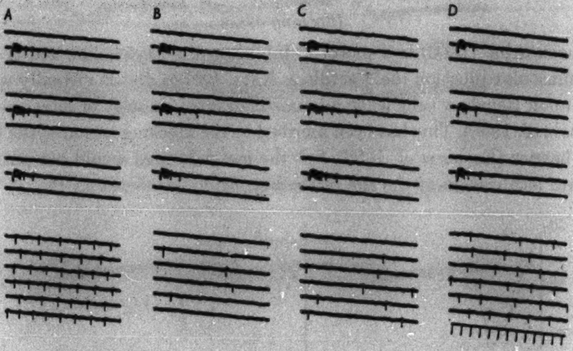

Fig. 4. In this figure, and in Fig. 5 *A–F*, each column from above downwards consists of responses to three separate ventral root volleys and the maximum discharge evoked by equal currents passed through a barrel containing acetylcholine. These responses were recorded on moving film, there being 5 msec between sweeps. *A*, control responses. DTC was then passed using a current of 3×10^{-8} A for 65 sec and the records *B*, *C* and *D* were taken 40, 60 and 180 sec, respectively, after this current commenced; time marker, 10 msec.

Unfortunately, the effect of DTC upon the synaptically evoked responses of this cell was not recorded. However, several other cells were tested in full, one of which is illustrated in Fig. 4. In any one column are shown responses to three separate ventral root volleys and below are the responses evoked by acetylcholine in a manner similar to that used for the tests of Fig. 3*A*. In Fig. 4, after the control records *A*, a current of 3×10^{-8} A flowed through the DTC barrel for 65 sec and the responses *B* and *C* were taken 40 and 60 sec after the onset of this current, while *D* was taken 2 min after its cessation. Although in *B* and *C* the response to injected acetylcholine was nearly abolished, there was very little alteration in the responses to synaptic excitation. Perhaps the last one or two spikes were not as regular in occurrence under the influence of DTC. A similar series is illustrated in Fig. 3*B* (○), where less DTC was used ($1\cdot5 \times 10^{-8}$ A for 75 sec). Again, the effect of directly applied acetylcholine was diminished. This same cell was tested with procaine, which was found by del Castillo & Katz (1957*a*) to be a powerful inhibitor of acetylcholine action at the motor end-plate of the frog. The experiment illustrated in Fig. 5 *A–F* is similar to that of Fig. 4, the upper three records showing the effect of synaptic stimulation of the cell and the lower record the result of a constant rate of electrophoretic injection of acetylcholine. Column *A* gives the control responses. A current of $1 \cdot 5 \times 10^{-8}$ A flowed through the procaine barrel for 60 sec and the records of *B* and *C* were taken 20 and 60 sec after this current commenced, and those of *D*, *E* and *F*, 20, 60 and 180 sec, respectively, after the current ceased. Procaine diminished the frequency of responses of the cell to acetylcholine and in *B* and *C* there was a definite reduction in the number of discharges evoked by synaptic stimulation. This series is plotted in Fig. 3*B* (●), where a comparison is made between the effects of both procaine and DTC upon the frequency of response evoked by constant rates of injection of acetylcholine. The procaine was slightly more rapid in action, but once the

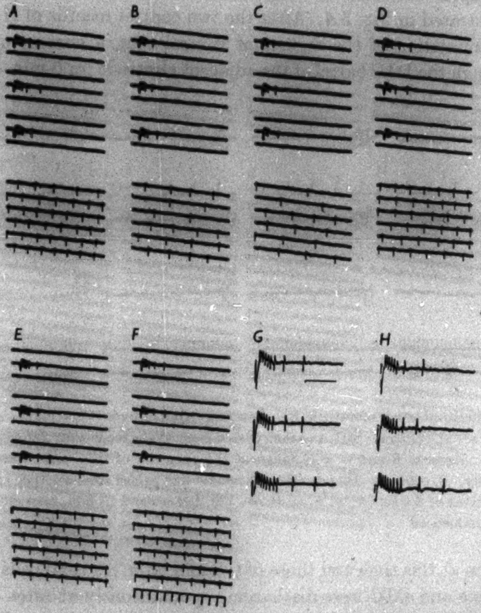

Fig. 5. *A–F* as for Fig. 4. *A*, controls. *B–F*, 20, 60, 80, 120 and 240 sec, respectively, after a current of $1 \cdot 5 \times 10^{-8}$ A commenced to flow through a barrel containing procaine for 60 sec. *G*, *H*, responses of a single Renshaw cell but evoked by maximal stimulation of the appropriate ventral root, *G* before and *H* 1 min after the administration of DTC 1 mg/kg intravenously. Time marker 10 msec.

drugs ceased to flow from the electrode the recovery phases were very similar. However, with both procaine and DTC, although some degree of reversibility occurred within 2–3 min, this was not complete. The effect of intravenously applied DTC upon a nearby cell in the same animal is shown in Fig. 5*G* and *H*. The number of responses evoked by synaptic stimulation was virtually unchanged 1 min after the injection of 1 mg/kg. Fig. 6*A*, *B* and *C* show records from a cell upon which DTC had no effect. This was from the same animal as the cell illustrated in Fig. 3*A*. After the two control records of *A*, evoked by maximal stimulation of the segmental ventral root, a current of 6×10^{-9} A flowed through the DTC barrel of the adjacent electrode for 5 min. The records *B* were taken at this time and those of *C* 2 min later. Several cells tested with both procaine and DTC were discharging spontaneously at rates of 8–50/sec. Both drugs depressed these responses by approximately the same factor by which the discharges due to applied acetylcholine were affected.

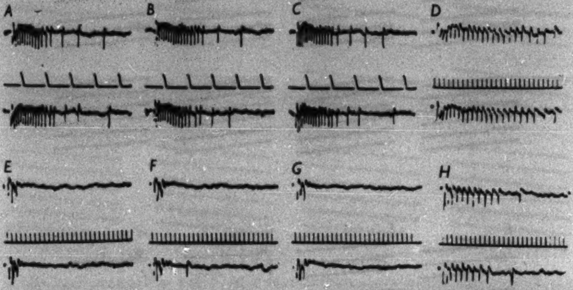

Fig. 6. Synaptic stimulation of a single Renshaw cell. Note different time marker, *A–C* 10 msec, *D–H* 1 msec. *A*, controls. DTC was then passed from an electrode with a current of 6×10^{-9} A for 5 min. Records *B* and *C* were taken at the cessation of this current and 2 min later, respectively. *D*, controls. Dihydro-β-erythroidine was passed from another barrel for 90 sec with a current of 2×10^{-7} A; *E*, *F*, *G*, *H*, 70, 120, 180 sec and 28 min, respectively, after this current commenced.

Dihydro-β-erythroidine (DHE) effectively blocks the effect of intra-arterially injected acetylcholine and nicotine upon Renshaw cells (Eccles *et al.* 1956; Curtis, Eccles & Eccles, 1957). However, when synaptic stimulation is used, the first two or three responses of the cell are relatively resistant to the drug, even when large doses are employed. This has suggested that the synaptic barrier gives a degree of protection to acetylcholine released inside it and that the initial high concentration of acetylcholine is sufficient to overcome the degree of post-synaptic block that can be established. When applied locally, DHE has a similar effect upon synaptically evoked responses. The repetitive responses of a Renshaw cell (same cell as Fig. 6*A*, *B*, *C*) are shown in Fig. 6*D–H*.

A current of 2×10^{-7} A flowed through the DHE barrel for 90 sec and the records *E*, *F*, *G* and *H* were taken at 70, 120, 180 sec and 28 min, respectively, after the onset of this current. There was a complete loss of the later responses of the cell, but in spite of the large amount of DHE used the first two responses persisted. The effect was reversible but recovery was incomplete at 28 min, which is further evidence that the dose was large. When smaller doses of DHE are used, the effect reverses more readily and fewer of the responses are blocked.

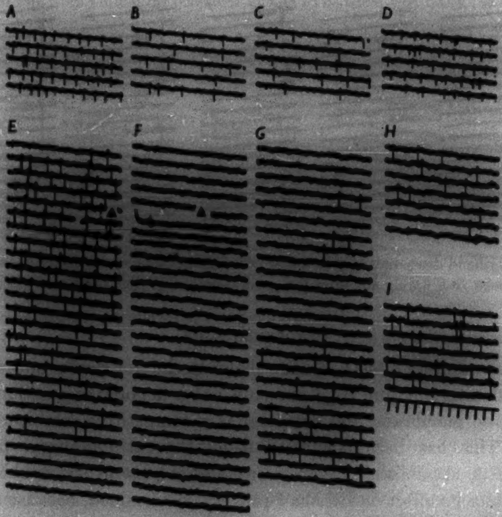

Fig. 7. *A–D*; a current flowed continuously through a nicotine electrode. After the control *A*, dihydro-β-erythroidine was passed from another barrel using a current of 8×10^{-9} A for 15 sec; *B*, *C* and *D* were recorded 10, 25 and 75 sec after this began. *E–I* (*E*, *F* and *G* being continuous); throughout this series a constant current passed acetylcholine into the neighbourhood of the same cell, the current having commenced several seconds before the beginning of *E*. At ▲ in *E*, 8×10^{-9} A was used to pass dihydro-β-erythroidine from the electrode until ▲ in *F*. *H* and *I* are respectively 18 and 30 sec after the cessation of the dihydro-β-erythroidine current.

Directly applied DHE blocks the effect of acetylcholine and nicotine injected from another barrel of the micro-electrode. Fig. 7 *A–D* shows this effect on a background discharge due to nicotine, and again it is reversible. Figs. 7 *E*, *F* and *G* are in continuity. A background discharge produced by acetylcholine was abolished within 2 sec by a current of 8×10^{-9} A through the DHE barrel (first triangle). Within 4 sec of the cessation of this current (triangle in Fig. 7 *F*), the cell again responded to acetylcholine (Fig. 7*G*). This recovery progressed slowly and was still incomplete in Fig. 7*I* after 30 sec. However, both the nicotine and acetylcholine series in Fig. 7 do not take account of the accommodation occurring when Renshaw cells are subjected to long applications of depolarizing drugs (Curtis & Eccles, 1958) and are of necessity incomplete. Nevertheless, they illustrate adequately the effect that DHE has on the responses of these cells to depolarizing agents.

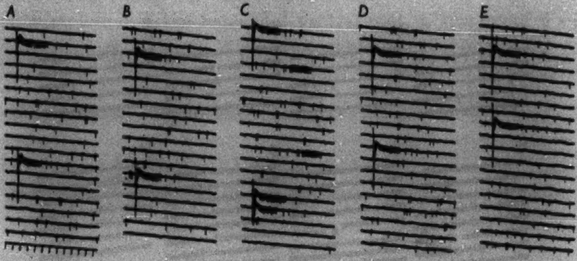

Fig. 8. Background discharges of a Renshaw cell, together with synaptically evoked responses. *A*, controls; *B–E* during and after the application of C_{10}; time marker 10 msec. See text.

Decamethonium

del Castillo & Katz (1957*b*, *c*) have shown in the frog nerve–muscle preparation that decamethonium (C_{10}) not only has a depolarizing action upon the post-synaptic membrane, but acts also as a competitive inhibitor of cholinesterase. This has been confirmed using the rat-diaphragm preparation (D. R. Curtis, unpublished observation), and in view of the double action of this substance its effect on Renshaw cells was determined, because it has been found to be inactive when administered intra-arterially. Fig. 8*A* shows the background discharge of a single Renshaw cell together with two series of repetitive discharges evoked by synaptic stimulation. Following this a current of 6×10^{-8}A flowed through the C_{10} barrel for 120 sec, and the subsequent records, taken in a similar fashion, were recorded at 30, 90, 150 and 210 sec after the onset of this current. There was a slight increase in the rate of background discharge, but very little alteration in the number of responses to synaptic nerve stimulation, such as would have been recorded if prostigmine had been used (Fig. 1). With the same cell, however, a more marked change was obvious when acetylcholine was used to increase the rate of discharge. In Fig. 9*A* the background discharge and five responses to stimulation of the ventral root are shown. In Fig. 9*B* acetylcholine was used to stimulate the cell and the current through this barrel ceased at the triangle. The response was relatively small and had virtually finished within 1–1·5 sec of the cessation of the current. C_{10} was then passed from the electrode with a current of 3×10^{-8}A and after 1 min the records *C* and *D* were taken, using the same conditions as those of *A* and *B*, respectively. There was a slight diminution in the background discharge and the responses to synaptic stimulation were slightly increased in the frequency of discharge and in its duration. However, the discharge rate produced by acetylcholine was increased by a factor of 1·5 and the effect was prolonged for many seconds after the acetylcholine had been turned off, at the triangle in Fig. 9*D*. These findings suggest that C_{10} has little action upon the acetylcholine released at the synapses, but behaves effectively as an anticholinesterase towards acetylcholine released from an electrode. This has been confirmed in many cells, another of which is shown in Fig. 10. In Fig. 10*A*, *B*, *C* and *D* are shown the responses to acetylcholine which in each case was passed by a current pulse of 30×10^{-9}A for 50 msec. Two seconds elapsed between sweeps, and each of the series of *A*, *B*, *C* and *D* are composed of three successive responses. After the three control responses *A*, a current of 7×10^{-9}A flowed through the C_{10} barrel while the responses in *B* were recorded, and was then turned off for the recording of *C* and *D*. The effect of acetylcholine was potentiated and prolonged by the action of C_{10}, which with these doses was readily reversible. However, with the same cell, very little effect even with large doses of C_{10} was observed upon the responses to synaptic stimulation. The three records of Fig. 10*E* are control responses and those of *F* were recorded after a time of 5 min during which a current of 5×10^{-8}A flowed through the C_{10} barrel. There was a little prolongation and intensification of action of acetylcholine released synaptically.

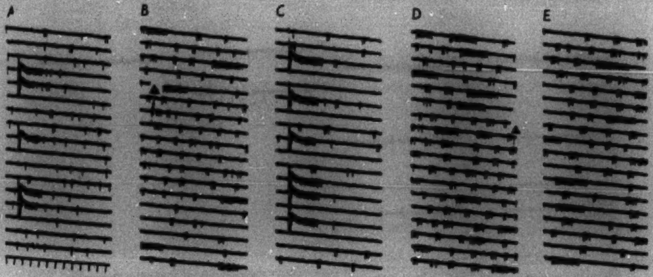

Fig. 9. *A*, background activity and synaptically evoked responses of a Renshaw cell. *B*, discharge produced by a current passing through the acetylcholine barrel which commenced 2 sec before *B* and ceased at ▲. *C*, as for *A*. *D*–*E* continuous, as for *B*. During *C*–*D*, C_{10} was passed from another barrel of the electrode, this current starting 60 sec before *C*; time marker, 10 msec.

On several occasions the effect of C_{10} on the discharge evoked by nicotine was determined. No prolongation of nicotine action occurred but there was on all occasions, with different cells in two animals, a definite reduction in the frequency of the response to nicotine and a small reduction in the duration of this excitation. This effect is illustrated in Fig. 11, where a comparison is made using the same cell between the effect of C_{10} on the frequency of the responses due to acetylcholine (●) and nicotine (○). This will be discussed later.

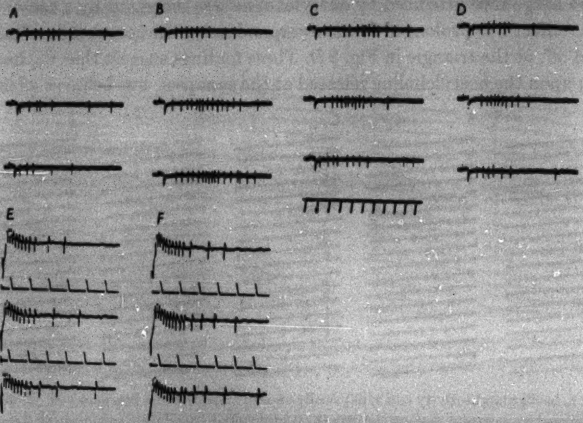

Fig. 10. *A–D*; each sweep shows responses evoked by a constant current pulse passing through an electrode containing acetylcholine; the records were taken in continuity, there being 2 sec between sweeps. After the three control records *A*, a current passed C_{10} from another barrel during the three records of *B*, this current ceasing just before the first record of *C*; time marker, 100 msec. *E*, *F*, synaptic stimulation of the same cell, *E* before and *F* after a current of 5×10^{-8} *A* was passed through the C_{10} barrel for 5 min; time marker, 10 msec.

DISCUSSION

The results reported in this paper are concerned both with the action of certain drugs on Renshaw cells and with the barriers surrounding these cells. It will be convenient to discuss these problems with the aid of a diagram (Fig. 12) which shows a single Renshaw cell (*RC*) in relation to both a multibarrelled electrode (*ME*) and a capillary (*C*) surrounded by the blood–brain barrier (*BBB*). The synaptic barrier (*SB*) surrounds the terminals of the axon collaterals (*AXC*).

Although it is clear that a 'blood–brain barrier' exists (Friedemann, 1942; Krogh, 1946; Tschirgi, 1952; Manery, 1954; Bakay, 1956; Dobbing, 1956; Davson, 1957), there is considerable confusion regarding its nature and site. It is possible that the barrier is related to capillary walls (Rodriguez, 1955) although histologically there is nothing to distinguish these capillaries from those elsewhere in the body (Patek, 1944; Woollam & Millen, 1954). Consequently, the barrier may be outside the actual vessel wall, being either the

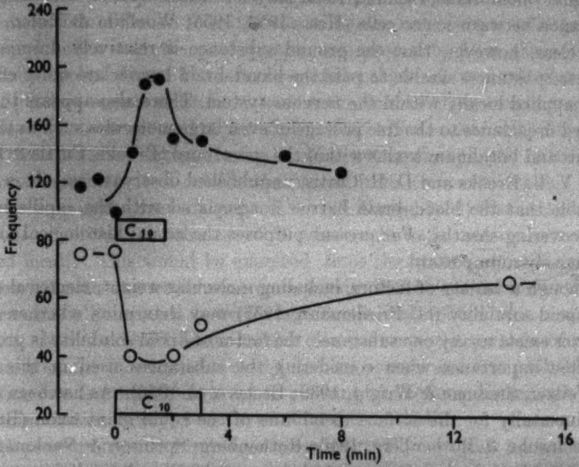

Fig. 11. The maximum frequency evoked by acetylcholine, ●, and nicotine, ○, when tested by using constant currents of either drug for 3–4 sec; note different frequency scales. As indicated, equal currents flowed through a C_{10} barrel from zero time. The two series were recorded 25 min apart from the same Renshaw cell.

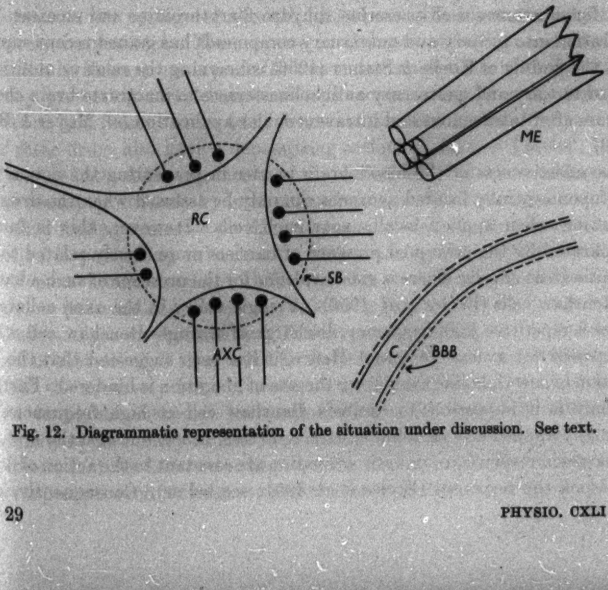

Fig. 12. Diagrammatic representation of the situation under discussion. See text.

'pia-glial' membrane (Tschirgi, 1952; Mayer & Bain, 1956) or even the ground substance between nerve cells (Hess, 1953, 1955; Woollam & Millen, 1954). It is clear, however, that the ground substance is relatively unimportant, because substances unable to pass the blood–brain barrier are quite effective when applied locally within the nervous system. There also appears to be no marked impedance to the free passage of even large molecules such as those of tetanus and botulinum toxins within the spinal cord (Brooks, Curtis & Eccles, 1957; V. B. Brooks and D. R. Curtis, unpublished observations.) It is highly probable that the blood–brain barrier is associated with the capillaries and their covering sheaths. For present purposes the actual histological location is relatively unimportant.

Although a variety of factors, including molecular weight, electrical charge and lipoid solubility (cf. Friedemann, 1937) may determine whether or not a barrier exists to any one substance, the factor of lipoid solubility is probably of prime importance when considering the substances used in this paper (Schweitzer, Stedman & Wright, 1939; Eccles *et al.* 1956). As has been shown experimentally for the surface membrane of the squid giant axon (Bullock, Nachmansohn & Rothenberg, 1946; Rothenberg, Sprinson & Nachmansohn, 1948), the blood–brain barrier might be expected to be relatively impermeable to quaternary ammonium compounds such as acetylcholine, prostigmine and tubocurarine. These substances occur only in ionic form and are therefore relatively insoluble in lipids. The salts of tertiary bases, however, are partly un-ionized and consequently are soluble in lipids. Thus no major barrier would exist for substances such as eserine, dihydro-β-erythroidine and nicotine. This separation into tertiary and quaternary compounds has gained recent support from the finding of Koelle & Steiner (1956) concerning the relative abilities of related tertiary and quaternary anticholinesterases to inactivate brain cholinesterase after intravenous and intraventricular application (cf. Mayer & Bain, 1956).

The effectiveness of the blood–brain barrier in preventing the action of a drug upon centrally located neurones can only be assessed when the drug has an action when applied locally near such cells. However, this is further complicated by the presumed presence of barriers more closely related to the synaptic areas of cells. There is good evidence for the presence of such a barrier for Renshaw cells (Eccles *et al.* 1956). A single volley in the axon collaterals causes a repetitive high-frequency discharge of a single Renshaw cell which may persist for at least 50 msec. Hence, it has been suggested that the free diffusion of acetylcholine away from the site of liberation is hindered. Further, not only is it impossible to excite a Renshaw cell at high frequencies by externally applied acetylcholine (Curtis & Eccles, 1958), but also the first two discharges in response to synaptic activation are resistant to the action of drugs that block the responses (Eccles *et al.* 1954; see below). Consequently, it is postulated that acetylcholine is released within a barrier which not only prevents its free diffusion from the site of release but also partially protects the acetylcholine and the post-synaptic receptor sites from the action of externally applied drugs. The behaviour of Renshaw cells towards pharmacologically active agents must be viewed in the light of these two barriers and the actions of the drugs used in this study will now be discussed with due regard to the two sites of liberation of acetylcholine: within the synaptic barrier from the nerve terminals, and outside the barrier from a micro-electrode. It has been reported in the previous paper (Curtis & Eccles, 1958) that acetylcholine is not as effective a depolarizer of Renshaw cells as is nicotine, even when applied locally. This would be expected, since the synaptic barrier prevents the free diffusion of acetylcholine from within. The occasional ineffectiveness of intra-arterially injected acetylcholine (Eccles *et al.* 1956; Curtis *et al.* 1957) suggests that the blood–brain barrier also prevents the passage of acetylcholine from the blood stream to the nervous system and it is probable that the same two factors are involved in blocking the actions of succinylcholine, carbaminocholine, acetyl-β-methylcholine and arecholine.

Both prostigmine and edrophonium have virtually no action upon Renshaw cells when administered intravenously, but are effective anticholinesterases when applied near a Renshaw cell. The responses to synaptic stimulation and to acetylcholine released externally to the synaptic barrier are reversibly intensified and prolonged, suggesting that cholinesterase both within the barrier and external to it is inactivated. Thus, this barrier offers little or no resistance to the passage of these drugs, whereas the blood–brain barrier is relatively impermeable. The increase in the rate of background discharge produced by these drugs is probably due to the inactivation of cholinesterase, thereby protecting acetylcholine released spontaneously from the terminals. However, the present series of experiments does not exclude the possibility that these drugs also have a depolarizing action (cf. Katz & Thesleff, 1957).

There is little difference between the action of DHE when administered either locally or diffusely by intravenous injection. The blood–brain barrier is therefore freely permeable to this substance. However, this barrier is very effective in preventing tubocurarine from reaching the nervous tissue after intravenous administration. Unfortunately, no assessment of the barrier offered to procaine administered systemically has been made, but even when applied locally both procaine and DTC are much less effective in blocking the action of acetylcholine than is DHE. Both have very little effect when tested against the relatively large concentrations of acetylcholine released by the nerve terminals, although they can diminish the frequency with which externally applied acetylcholine can discharge a single Renshaw cell. It also is of interest that the first two or three responses to orthodromic stimulation are not suppressed even by large concentrations of DHE.

All three drugs, DTC, DHE and procaine have a curare-like action at the motor end-plate (Harvey, 1939; Unna, Kniazuk & Greslin, 1944; del Castillo & Katz, 1957*a*) and it would be expected that each would produce an inactive receptor–drug complex preventing access of acetylcholine to the post-synaptic receptors. An explanation is required for the fact that all three drugs can abolish the responses of a cell due to acetylcholine applied outside the synaptic barrier, and yet differ in their actions upon responses due to acetylcholine released from the nerve terminals. If this differential effect was due solely to a local competition between blocking agent and acetylcholine for the receptor site, it might be expected that the effectiveness of a blocking agent would depend upon the relative stability of the respective receptor–drug complexes (cf. del Castillo & Katz, 1957*a*). However, this is unlikely for two reasons. As pointed out by del Castillo & Katz (1957*a*), the dissociation of these blocking agent–receptor complexes appears to be relatively slow when compared with action of acetylcholine and it is unlikely that enough drug could be displaced in the time involved. Secondly, the rates of decay of the inhibitions of acetylcholine action by DTC, procaine and DHE when assessed at the end-plate regions (del Castillo & Katz, 1957*a*; D. R. Curtis, unpublished observation) suggest that the DTC complex is more stable than either of the other two and that the DHE complex is relatively unstable. However, the results reported here show definitely that the blocking action of DHE upon Renshaw cells, when tested by high concentrations of acetylcholine released within the synaptic barrier, is much greater than that observed with DTC and procaine. Thus the relative actions of these three drugs may depend upon the permeability of the synaptic barrier to each (cf. del Castillo & Katz, 1957*a*, p. 351). It can be postulated that the barrier is relatively permeable to DHE, and less so to procaine and DTC. Enough of these agents penetrate to block the responses due to randomly released acetylcholine from the terminals and to the relatively small amount of acetylcholine which penetrates the synaptic barrier when injected from an electrode. However, when large quantities of acetylcholine are released from the nerve terminals, insufficient post-synaptic receptor sites are occupied by DTC or procaine to cause a significant depression, whereas more are occupied by DHE. Within the barrier the relative stability of the drug–receptor complex may be important, because with the large doses of DHE used in this study it can reasonably be expected that all post-synaptic receptors might be occupied, and this block is overcome by the high acetylcholine concentration. This point might be further elucidated at the neuromuscular junction.

Decamethonium is of considerable interest because of its behaviour both as a depolarizing agent and as an anticholinesterase (Paton & Zaimis, 1949; del Castillo & Katz, 1957*c*). When injected intra-arterially, doses of 100 μg do not fire Renshaw cells (Eccles *et al.* 1956), but this test cannot be used as evidence that the blood–brain barrier is impermeable to this drug. When applied from a micro-electrode C_{10} has very little effect upon the discharge of a Renshaw cell evoked synaptically. Consequently, it can be assumed that very little passes the synaptic barrier to inhibit the action of cholinesterase located near the post-synaptic receptor sites. On the other hand, however, the effect of acetylcholine applied externally to this barrier is both accentuated and prolonged, indicating that C_{10} effectively inhibits the action of cholinesterase located in this region. This finding is not explicable by the differential inhibition of specific and non-specific cholinesterase by C_{10}, because C_{10} has a greater inhibitory action upon true cholinesterase (Paton & Zaimis, 1949) which is presumably in higher concentration within the synaptic barrier than external to it. No evidence has been obtained that C_{10} can depolarize Renshaw cells sufficiently to cause discharges. The slight increase observed in the rate of background discharge is suggestive, but could be due to the anticholinesterase action of the drug in protecting acetylcholine released spontaneously from the nerve terminals. The finding that the rate of discharge produced by nicotine is diminished in the presence of C_{10} suggests that C_{10} occupies post-synaptic receptor sites and so blocks the action of nicotine (cf. del Castillo & Katz, 1957*c*). However, it is also possible that C_{10}, by indirectly facilitating the action of the quantally released acetylcholine, could produce the same result by decreasing the sensitivity of the post-synaptic receptors. Unfortunately, no assessment has been made of the effect of the blood–brain barrier upon the movement of C_{10} from the blood stream to nervous tissue. The experiments cited (Eccles *et al.* 1956) were not designed to detect the effect of C_{10} upon cholinesterase and the lack of a direct action of the drug upon Renshaw cells has been sufficiently explained by the synaptic barrier. However, it is possible that the blood–brain barrier is impermeable to C_{10}, because Irwin & Wells (1957) have been unable to demonstrate an action of succinylcholine, C_{10} and DTC upon the central mechanisms controlling respiration—unless, indeed, these neurones are surrounded by a synaptic barrier.

It can be concluded from the results reported in this and the previous paper (Curtis & Eccles, 1958) that the anomalous behaviour of Renshaw cells when tested with intravenously and intra-arterially injected drugs (Eccles *et al.* 1954; Eccles *et al.* 1956) is not due to essential differences between the cholinergic synapses formed by the motor axon or its branches centrally and peripherally, but to the presence of diffusional barriers existing between the central site of release and action of acetylcholine and the sites of application of the testing drugs. It has thus been shown that acetylcholine is the chemical transmitter released at the synapse between the axon collaterals and Renshaw cells, just as it is also released at the neuromuscular junction. When the actions of drugs upon neurones are being considered, care must be taken concerning the relative permeabilities of diffusional barriers that might exist between the site of application and the presumed sites of action. The blood–brain barrier could hinder the free access of intra-arterially and intravenously injected drugs to centrally located nerve cells. In addition, however, it is probable that the synaptic regions of many neurones are surrounded by a barrier, because repetitive discharges at high frequency in response to a single presynaptic stimulus are observed for many types of neurones (cf. McIntyre, Mark & Steiner, 1956). The simplest explanation of this involves the limitation of the free diffusion of the chemical transmitter away from its site of liberation.

The characters of the two barriers investigated in this paper are entirely different. Whereas lipoid solubility may be a factor in determining the passage of a substance across the bloo–brain dbarrier, substances such as prostigmine are freely diffusible across the synaptic barrier. It appears that drugs of high molecular weight have difficulty in reaching the synaptic areas upon Renshaw cells, since the barrier exists to maintain a high concentration of acetylcholine at the post-synaptic receptors. The possibility that the barrier excludes true cholinesterase from the locality of the synapses is rendered untenable by the finding that some anticholinesterases (prostigmine) prolong the discharge evoked by synaptic action, whereas C_{10} has little effect upon this type of response. Consequently, prostigmine must inactivate cholinesterase located within the synaptic barrier.

SUMMARY

1. The pharmacological behaviour of single Renshaw cells has been tested by passing drugs electrophoretically from an adjacent multibarrelled electrode, the responses of the cell being recorded from one of the barrels.

2. Some drugs such as prostigmine, edrophonium (Tensilon), procaine, tubocurarine and decamethonium, which are relatively ineffective when administered intravenously or intra-arterially, become much more effective when applied near such cells.

3. All evidence confirms the cholinergic nature of transmission between motor axon collaterals and Renshaw cells.

4. Both the blood–brain barrier and another diffusional barrier, more closely related to the synaptic terminals upon Renshaw cells, can prevent drugs from reaching the site of release of acetylcholine and the underlying post-synaptic receptors.

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MEMBRANE POTENTIALS RECORDED WITH HIGH-RESISTANCE MICRO-ELECTRODES; AND THE EFFECTS OF CHANGES IN IONIC ENVIRONMENT ON THE ELECTRICAL AND MECHANICAL ACTIVITY OF THE SMOOTH MUSCLE OF THE TAENIA COLI OF THE GUINEA-PIG

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Membrane potentials of the rhythmically active smooth muscle of the taenia coli of the guinea-pig have been described previously by Bülbring (1954, 1955, 1956, 1957). The effects of changes in ionic environment on the mechanical activity of intestinal muscle (rate of spontaneous contractions and tonus) have been studied by Evans (1926), Magee & Reid (1927), Vogt (1943) and Ambache (1946). Less is known of the electrical activity under these conditions. Effects of changes in NaCl concentration have been described by Holman (1957*a*) and a brief account has been given of observations when the KCl concentration was varied (Holman, 1957*b*). Burnstock & Straub (1958) studied the effects of changes in the concentration of K, Ca and Cl ions on the resting potential of strips of smooth muscle from the gut of various species, using the sucrose-gap method. In general their results were similar to those found with intracellular recordings.

In the present experiments relatively high resistance capillary micro-electrodes have been used to record membrane potentials in an attempt to minimize the degree of cell injury. Some aspects of the normal activity recorded with these electrodes will be described, together with the effects of variation in the concentration of K, Cl and Ca ions. Some further experiments have been carried out in solutions containing very low concentrations of Na ions.

METHODS

The method of mounting small lengths of taenia coli (4–6 mm) and the constant-temperature bath (36° C) have been described previously (Bülbring, 1954, 1955; Holman, 1957*a*). The input stage, amplifier and camera were conventional. The input time constant was measured at frequent intervals with the tip of the micro-electrode just touching the surface of the bathing solution. The input capacity was from 1–2 pF. The upper surface of the taenia was never more than 2 mm below the surface of the bathing solution which flowed continuously. Tension was measured with a mechano-electronic transducer valve (RCA 5734) mounted in the manner described by Bülbring (1955).

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* Melbourne University Travelling Scholar.

The resistance of the micro-electrodes varied from 35 to 70 MΩ. Only electrodes with tip potentials of less than 5 mV were used for determination of the resting potential. Electrodes with higher tip potentials were used in some experiments where the spike configuration was studied, provided the tip potential and the resistance of the electrode were steady.

The normal solution used in all experiments except those with low Na solutions contained (mM): NaCl 134, KCl 4·6, $MgCl_2$ 0·1, $NaHCO_3$ 16·3, NaH_2PO_4 1·1, $CaCl_2$ 2·5, glucose 7·8; and was aerated with CO_2 5 %, O_2 95 %. In the experiments with excess KCl, solid KCl was added to this normal solution, unless otherwise stated in the text. The 'sulphate' solution was prepared by replacing the NaCl and KCl present in the normal solution with an equivalent amount of Na_2SO_4 and K_2SO_4. Since the amount of $CaCl_2$ was unchanged, the concentration of ionized Ca was reduced.

The K-buffered 'normal' solution which was used in the low Na experiments contained (mM): NaCl 150, $KHCO_3$ 5·0, aerated with CO_2 1 %, O_2 99 %; the concentrations of $CaCl_2$, $MgCl_2$ and glucose were as before. Choline chloride was used to replace NaCl.

RESULTS

Normal activity

Since during spontaneous activity the membrane potential of the taenia coli is never steady, it is necessary to specify what is meant by the term 'resting potential'. Throughout the present experiments the maximum level of polarization has been measured, whether this was reached by the repolarization phase of the spike or at a later stage during the interval between spikes. This will be called the resting potential (in accordance with previous writers), although it is realized that 'maximum polarization' would be a more accurate expression. Fig. 1 shows the distribution of values of the resting potential, recorded during spontaneous activity from 105 different fibres in 24 preparations. The mean value was 51·5 mV (S.E. = 0·36). During pauses between bursts of spontaneous activity higher potentials up to 70 mV were recorded.

The simplest spikes ranged from 50 to 75 mV in amplitude. Typical records are shown in Fig. 2. The lower trace was photographed on a slow time base and shows the relation between the spikes and the slower fluctuations of membrane potential which occurred during the interval between spikes. Although the spikes arose quite steeply from the fluctuating base line, their initial phase (or 'foot') was relatively slow. Repolarization led into a brief 'after-positivity' (this term being taken over from the usual nomenclature dealing with surface p.d.) but in this fibre the maximum level of polarization was only reached about 400 msec later. The upper trace was photographed using a fast repetitive sweep and the slower fluctuations in the resting potential were therefore obscured. The initial phase of the spike can be seen, leading into the faster component. Repolarization led to an after-positivity which carried the membrane potential to a level of polarization greater than that

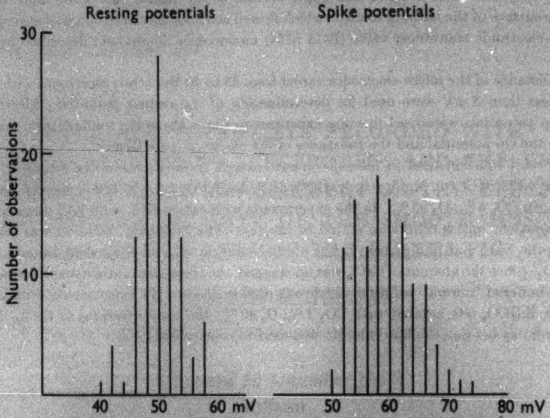

Fig. 1. Distribution of values for the resting potential and the spike height measured during spontaneous activity in normal solution.

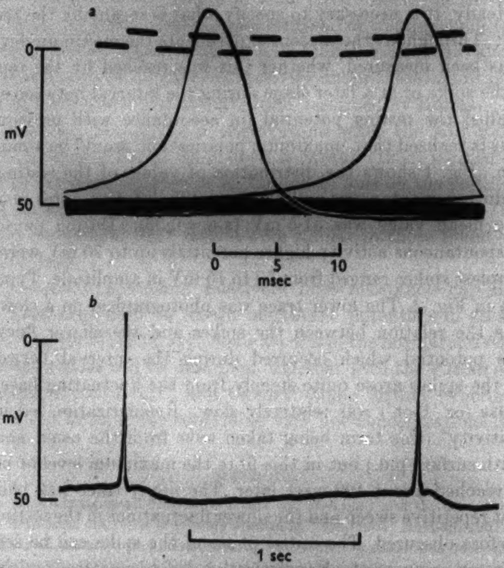

Fig. 2. Typical records of spikes recorded during normal spontaneous activity: *a*, swept time base; *b*, stationary trace, moving paper.

reached during the pause between spikes. The maximum rates of rise and decay were 18 and 15 V/sec respectively and there was an overshoot at the peak of 12·5 mV. These spikes were recorded from a fairly active preparation and the repolarization phase showed a slight delay before reaching the maximum rate. In less active preparations the upper part of the spike was more nearly symmetrical. The time course of the repolarization phase was plotted logarithmically for several different spikes (logarithm of voltage against time).

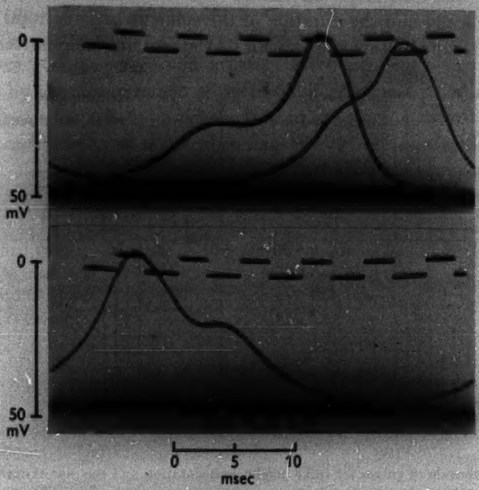

Fig. 3. Typical records of spikes of a complex type recorded during spontaneous activity; swept time base.

In every case the graph was non-linear, the rate of decay being slower near the peak of the spike, and faster at the foot, than that of an exponential decay with a single time constant.

Not all the spikes recorded during spontaneous activity showed this con-configuration. Although some fibres showed consistently regular activity, made up of spikes like those in Fig. 1, in other fibres such spikes were intermingled with potentials of a complex shape. (See normal records in Fig. 7.) These potentials were always smaller than the simple spikes. The largest of them showed notches on the depolarization phase or on the repolarization phase or on both phases (Fig. 3). The smaller they became, the greater was the irregularity of their shape. In many records they appeared to consist of an initial phase which was slower and more prolonged than that of the larger spikes, with a faster and highly variable component arising from it. The smallest 'spikes' showed no indication of a fast component. A possible explanation for these potentials is discussed in a later paper (Bülbring, Burnstock & Holman, 1958).

In one series of experiments, spikes were recorded on a fast time base, as is shown in the upper part of Figs. 2 and 3. Individual spikes were selected which showed no discontinuities during the rising or falling phase (like those in Fig. 2). The total height from the peak of the spike to the point of maximum polarization was measured. Maximum rates of rise and decay were determined graphically and the duration of the spike at half the total height was noted. The results of this analysis for 144 spikes are summarized in Fig. 4.

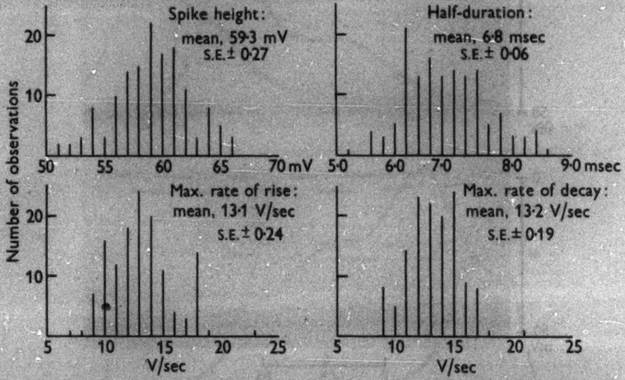

Fig. 4. Distribution of values for spike height, half-duration and the maximum rates of rise and decay for a series of spikes recorded during spontaneous activity.

The height of the spikes selected in this way showed a fairly normal distribution. When the maximum rates of rise and decay were plotted against height of spike, both graphs showed a wide scatter. Visual observation suggested that there was a trend towards faster rates of rise, but not towards faster rates of decay, with increasing height of spike. There was no correlation between the maximum rate of rise and the resting potential, though there was some indication that the maximum rate of rise was greatest when the duration and amplitude of the initial phase of the spike was small.

Slow rhythmic fluctuations in the resting potential were present to a varying degree in all records, both during and in the absence of spikes (see Bülbring, 1957). The amplitude of these slow waves varied from less than 1 to 10 mV and their duration was from 0·5 to 1 sec (see Fig. 2, lower trace and normal records in Figs. 7, 13 and 14). During regular activity they were synchronous with the spikes. Comparison of many records, however, showed that there was no consistent level of depolarization from which the spikes arose. On some occasions the initial phase of the spike was continuous with the slow wave so that the spikes showed pronounced prepotentials and appeared to have been initiated by the slow waves close to the point of impalement. The possible role of the slow waves in the initiation of the spike discharge is discussed in another paper (Bülbring *et al.* 1958).

The frequency of the spike discharge in many fibres was inversely proportional to the resting potential, in accordance with the observations of Bülbring (1955). In some fibres, however, fluctuations in frequency occurred in the absence of changes in resting potential. Unless the activity was highly irregular there was a close correlation between spike frequency and the tension maintained by lengths of taenia of up to 6 mm. In most records each spike was associated with a definite contraction in the tension record, which suggested that the activity of many fibres must have been synchronous.

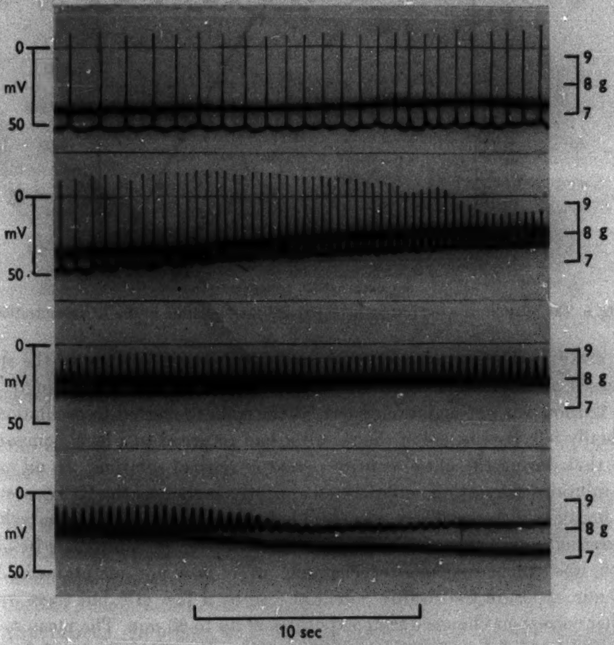

Fig. 5. Continuous record from a single fibre illustrating the changes in electrical and mechanical activity as the KCl concentration of the bathing solution was changing from normal to twenty times normal (20 × KCl). The continuous dark line is the record of tension.

Changes in KCl concentration

Excess KCl. The changes in electrical and mechanical activity of the taenia coli resulting from an increased KCl concentration are summarized in Fig. 5. This is a continuous record from an impalement which lasted while the concentration of KCl was changing from normal (left-hand side of the top row) to twenty times normal (henceforth 20 × KCl). The resting potential declined rapidly at first and then more gradually. The spikes continued to overshoot until the resting potential had fallen to about 35 mV. Beyond this point peaked negative waves were recorded, whose amplitude declined until they died away in the form of a damped oscillation. The frequency of the spikes increased as the resting potential fell and the tension rose accordingly. The high tension was not maintained but, as the spikes reverted to oscillations, it gradually fell. By the end of the record it had returned to a level comparable with that during the initial control period in normal solution. In all excess KCl solutions the resting potential fell abruptly as soon as the KCl concentration began to increase. There was some indication that the minimum value of the resting potential in very high KCl concentration (greater than 10 × KCl) which was reached soon after changing over, was not maintained. After 7–10 min exposure the resting potential rose by a few mV, but there was no further consistent change during exposures of up to 60 min. The mean resting potential for different KCl concentrations was therefore calculated from all impalements made after the first 12 min.

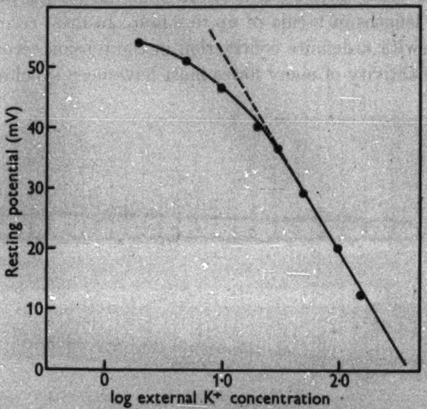

Fig. 6. The relation between resting potential and the logarithm of the K concentration.

Fig. 6 shows the graph obtained by this procedure. As the KCl concentration was increased up to about 30 mM the depolarization increased slowly. Higher concentrations caused larger depolarizations and a linear relation was established. In the graph the slope of the line was 33 mV/tenfold change in concentration. In a shorter series of experiments the added KCl replaced an equivalent amount of NaCl. The mean resting potentials for different concentrations of KCl were slightly lower than before but the differences were very small. In 4 × KCl (replacement) the mean resting potential was 38·0 mV (S.E. = 0·37) compared with 39·8 mV (S.E. = 0·37) when solid KCl was added to the normal solution. In 30 × KCl (replacement) the mean resting potential was 10·0 mV (S.E. = 0·40) compared with 12·1 mV (S.E. = 0·47) (addition).

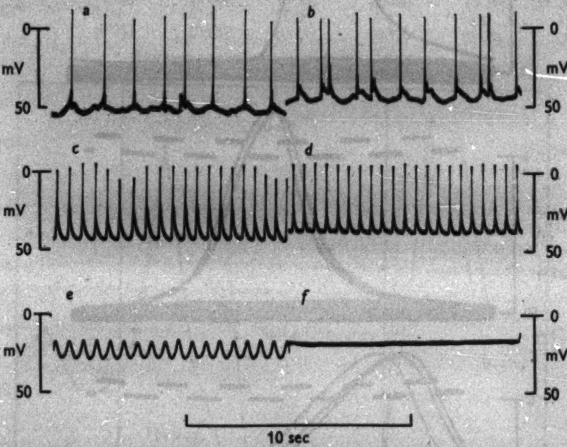

Fig. 7. Electrical activity recorded in: *a*, 1 × KCl; *b*, 2 × KCl; *c*, 4 × KCl; *d*, 6 × KCl; *e*, 10 × KCl; and *f*, 20 × KCl. Records taken after 12 min exposure to the excess KCl solutions: all from different preparations except *a* and *b*.

Some typical records of the electrical activity recorded after prolonged exposure (greater than 12 min) to different KCl concentrations are shown in Fig. 7. The traces taken in 1 × KCl and 2 × KCl came from the same experiment. Though the resting potential was decreased and the spike frequency increased in 2 × KCl, there was little change in the pattern of the discharge. As the concentration was increased to 4 × KCl and 6 × KCl the spike height decreased. In 10 × KCl a maintained oscillation like that in Fig. 7 was seen in about half the preparations. In others, a series of damped oscillations occurred, or, after the initial oscillation had died away, the resting potential was undisturbed. No oscillations were recorded after a few minutes exposure to 20 × KCl.

To determine the details of the configuration of the spikes in 2 × KCl and

4 × KCl records were taken on a fast time base. As in previous experiments of this type, only the simplest spikes were analysed. Some typical records are shown in Fig. 8 and the results are summarized in Table 1. The fall in spike height in 2 × and 4 × KCl could be accounted for by the lower resting potentials, and the overshoot was unchanged. The maximum rates of rise and decay were not affected by 2 × KCl; in 4 × KCl, however, the maximum rate of rise was reduced to about half that of normal and the maximum rate of decay to about one third.

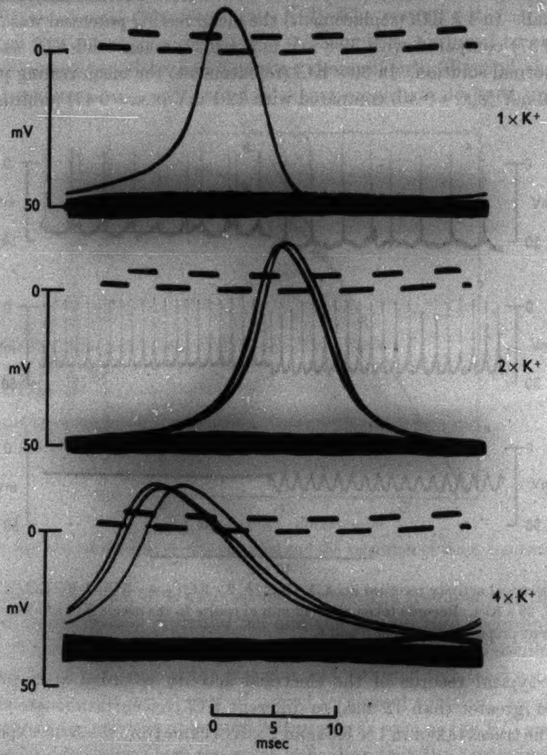

Fig. 8. Spikes in 1 × KCl, 2 × KCl and 4 × KCl. Records taken after 20 min exposure to the excess KCl solutions: from the same preparation.

The changes in spike frequency and tension which occurred during and after exposure to a solution containing 10 × KCl are shown in Fig. 9. In this experiment the tension rose at first in accordance with the increased spike frequency, reaching a maximum value 0·7 g higher than the control level. The frequency of the discharge remained high but the tension fell. During this period the spikes changed into small oscillations which eventually died away after about 20 min exposure. In 4 × KCl and 6 × KCl spike frequency and tension were elevated throughout the exposure period. Concentrations of KCl which abolished the spike discharge cause a fall in tension to a level below that of the control period. The fall in 30 × KCl was more pronounced if the NaCl had been replaced by KCl.

TABLE 1. Effect of KCl concentration on spike configuration

Concentration	Resting potential (mV)	Spike height (mV)	Over-shoot (mV)	Max. rate of rise (V/sec)	Max. rate of decay (V/sec)	Half-duration (msec)
1 × KCl	49·1	60·6	11·9	14·5	12·4	6·9
	(0·31)*	(0·37)	(0·42)	(0·32)	(0·30)	(0·09)
2 × KCl	45·4	56·8	12·1	15·6	12·1	6·4
	(0·49)	(0·36)	(0·36)	(0·28)	(0·29)	(0·12)
4 × KCl	38·5	51·4	12·9	8·4	4·1	12·7
	(0·27)	(0·25)	(0·27)	(0·11)	(0·08)	(0·23)

* Numbers in parentheses indicate S.E. of mean.

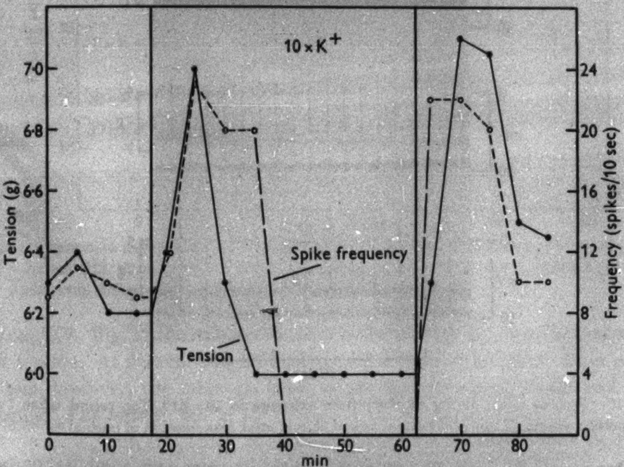

Fig. 9. Changes in spike frequency, ○, and tension, ●, during an experiment with 10 × KCl. The excess KCl was introduced at 15 min and the normal solution readmitted at 60 min. Each point represents the average of all readings taken during a period of 5 min.

Changing over to normal solution caused an immediate increase in spike frequency and tension. This increase was small after exposure to 4 × KCl or 6 × KCl since the spike frequency and tension were already high. The effect was quite dramatic if the muscle had been exposed to higher concentrations. In Fig. 9 for example, the contraction which occurred on washing out was greater than the initial contraction in response to the excess KCl. The duration of this period of increased activity depended on the previous concentration of KCl. After removal of the lower concentrations it was brief and after a few minutes the muscle relaxed to a level well below that of the control. If the concentration of KCl had been high, frequency and tension returned to normal more slowly and gradually: the final relaxation was less well marked or absent altogether.

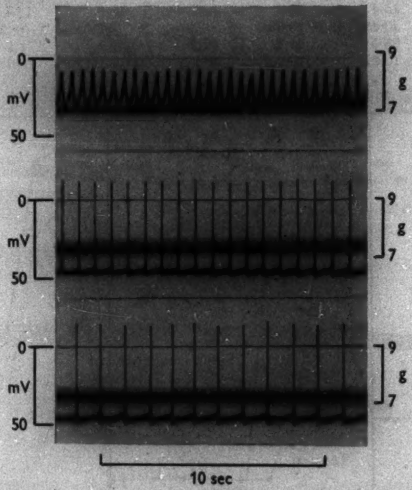

Fig. 10. Records taken during recovery from exposure to 20 × KCl. Top record taken 7 min, middle record, 23 min and bottom record 28 min after readmission of normal solution.

The records shown in Fig. 10 were taken during this delayed recovery from exposure to 20 × KCl. The first trace shows peaked negative waves which were similar to those recorded at the same resting potential when the KCl concentration in the bath was increasing (see Fig. 5). The second trace was taken 16 min later. The frequency of the spikes was still high but they had returned to a normal configuration. The last trace was taken from the same impalement 5 min later when the frequency was comparable with that of the initial control period (in normal solution).

Fig. 11 shows the time course of the changes in resting potential which occurred during the recovery period. The initial rate of increase after return to normal solution was very similar for all previous concentrations of KCl.

After removal of 30 × KCl the mean resting potential rose to 37 mV in the first 10 min. After this initial rapid increase, however, the resting potential rose much more slowly, and 20 min later it had only reached 45 mV. The effects of all excess KCl solutions were completely reversible and from 40 to 60 min after removal of 30 × KCl solutions the resting potential and the spike discharge returned to normal.

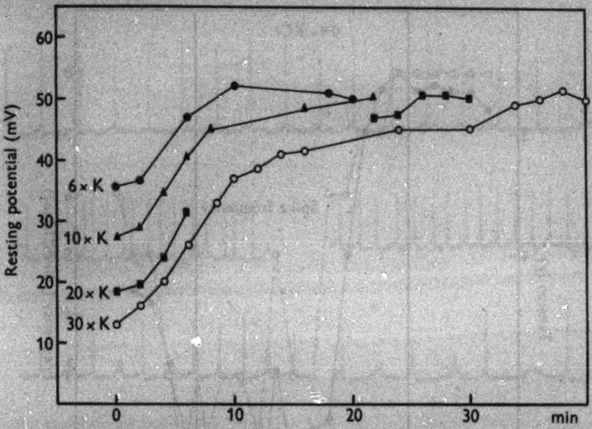

Fig. 11. Changes in resting potential during recovery in normal solution after previous exposure to excess KCl. Each point represents the average of all readings taken during a period of 2 min, from different preparations.

Low KCl. Fig. 12 illustrates the effect of 0·4 KCl (2 mM) on spike frequency and tension. At first the low KCl solution had little or no effect. After about 15 min, however, the intervals between the spikes became longer and very irregular. The tension fell in accordance with the lower frequency of spikes. After washing out, frequency of spikes and tension increased rather slowly so that 30 min later they were still lower than normal.

The irregular activity and low tension which occurred during prolonged exposure to the low KCl solutions were associated with a great deal of movement, and it was difficult to obtain good impalements. The mean resting potential after 12 min exposure was 54 mV—very slightly higher than in normal solution. No significant changes in shape of spikes were observed, although there seemed to be a predominance of complex potentials.

The effect of complete removal of K ions was observed in one experiment. An initial period of somewhat increased activity was seen which lasted for about 10 min. During this period the resting potential was slightly reduced (mean value 46 mV) but large spikes were recorded (59–64 mV), which showed a very prominent after-positivity. After 20 min exposure frequency of spikes and tension became very irregular and the records were similar to those taken in 0·4 × KCl. After 35 min the spikes ceased altogether. The tension was low and steady (2 g below the initial control level). The mean resting potential for the period after cessation of the spikes was 66 mV. After washing out, the resting potential remained high (greater than 60 mV) for 20 min. The tension was unchanged until the first waves of contraction appeared 35 min after washing out. About this time spikes were recorded again and the frequency and tension gradually increased to their normal levels.

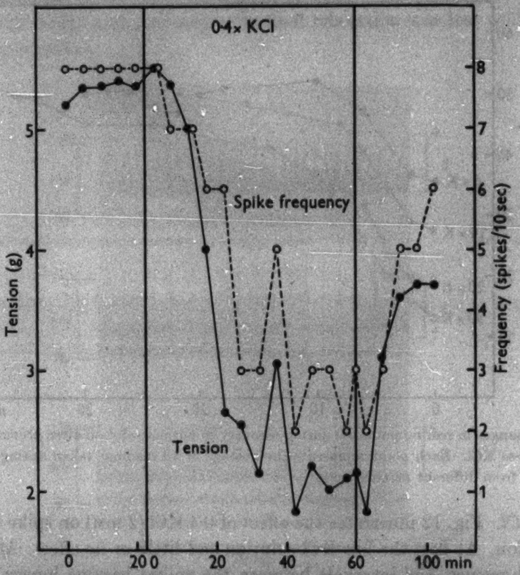

Fig. 12. Changes in spike frequency, ○, and tension, ●, from an experiment with 0·4 × KCl. The low KCl solution was introduced at 0 min and normal solution readmitted at 60 min. Each point represents the average of all readings taken during periods of 5 min.

Replacement of Cl with SO_4. Immediately after changing over to the sulphate solution the resting potential fell and there was an increase in spike frequency and tension. During the experiment illustrated in Fig. 13 the tension rose from a control level of 7·4 g to 8·6 g and it remained high for about 15 min. The maximum frequency in this case was 1·7/sec. The highest frequency noted in all four experiments was 2·6/sec and the largest increase in tension was 1·25 g. This period of excitation lasted from 15 to 20 min. If the muscle was left in the sulphate solution for longer periods, the interval between the spikes lengthened and the spikes became irregular. Records taken after 30 min exposure showed an irregular low-frequency discharge which was somewhat similar to that of the control period. The number of spikes showing definite pre-potentials, however, was higher than in normal solution and slow waves were frequently recorded in the absence of spikes.

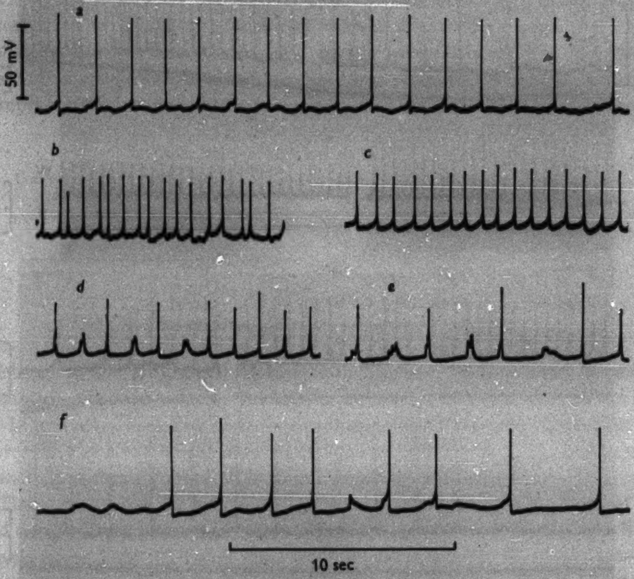

Fig. 13. Records from an experiment with sulphate solution (see Text). *a*, was taken in norma solution; *b*, 7 min; *c*, 10 min; *d*, 17 min; *e*, 21 min; and *f*, 28 min after exposure to the sulphate solution.

The effects were studied of 6 × K and 30 × K in sulphate solution. The initial increases in frequency of spikes and in tension were similar to those caused by the excess K in chloride solution. In 6 × K (sulphate) the discharge continued throughout the period of exposure and the tension remained high. In 30 × K, after cessation of the spikes the muscle relaxed very abruptly to a level which was considerably lower than that reached after prolonged exposure to 30 × K in chloride solution. Recovery after removal of 30 × K was similar for both chloride and sulphate solutions. After washing out 6 × K (sulphate), however, there was no indication of an increase in frequency of spikes or in tension.

Soon after the normal K concentration had been fully established, the spikes ceased and the tension fell.

Records from an experiment with 6 × K (sulphate) are shown in Fig. 14. After prolonged exposure the discharge consisted of peaked negative waves,

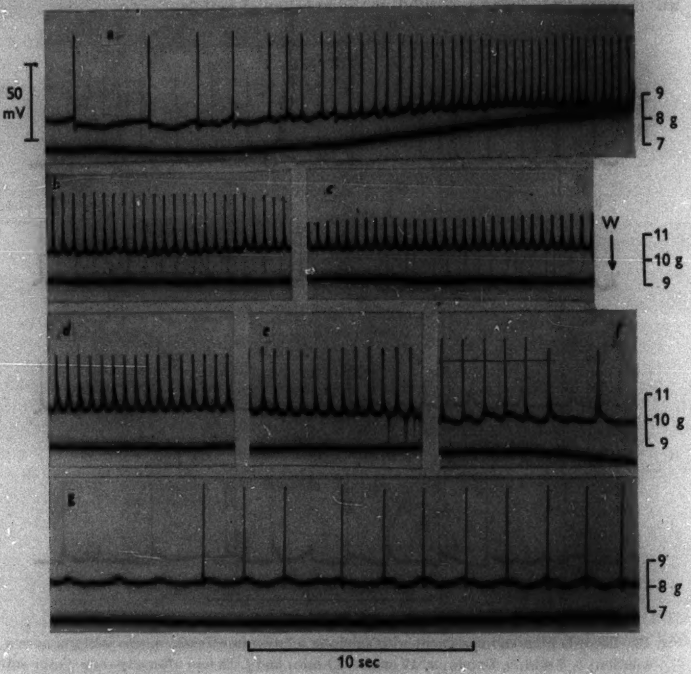

Fig. 14. Records illustrating the effect of 6 × K in sulphate solution. *a*, continuous record showing changes in activity as the excess K solution entered the bath; *b*, records taken after 5 and *c*, 20 min exposure to the excess K solution; *d*, *e*, *f*, records taken 2, 3 and 4 min after readmission of normal solution; *g*, continuous record showing return of activity in normal solution after a pause, following *f*, of 7 min.

without overshoot (see last trace before washing out). The amplitude of these waves was much smaller than that of the potentials recorded after prolonged exposure to 6 × K in chloride solution (compare Fig. 7). The cessation of the discharge soon after washing out is also shown in Fig. 14. In this experiment the pause in activity lasted for 7 min, after which normal activity was resumed (lowest trace).

The resting potential after 12 min exposure to 30 × K (sulphate) was reversed by a few mV (mean = 2·7 mV, inside positive, S.E. = 1·83). The resting potential in 6 × K was also lower in sulphate than in chloride solution (28·9 mV, S.E. = 1·45 compared with 36·7 mV, S.E. = 0·76), which might have been expected from the nature of the discharge in the sulphate (6 × K) solution.

Very low Na concentrations

The effect of very low Na concentrations on the spike configuration was reinvestigated with high-resistance electrodes and a fast time base. As in previous experiments, only the simplest spikes were analysed. The K-buffered 'normal' solution was used and the NaCl was replaced with choline chloride. Atropine (10^{-4}) was added to all solutions. This did not affect the spontaneous discharge in the K-buffered 'normal' solution, nor did it prevent the initial excitation due to the choline chloride in the low-Na solutions. During the first 10–15 min after changing over to a choline solution the spike frequency was increased, the spike height reduced and the spike duration increased. In most cases only spikes recorded after 20 min exposure were analysed.

TABLE 2. Effect of very low Na on spike configuration

Concentration (mM)	Spike height (mV)	Half-duration (msec)	Max. rate of rise (V/sec)	Max. rate of decay (V/sec)
20	61·6	9·3	10·4	9·8
	(0·38)*	(0·12)	(0·23)	(0·12)
10	53·2	15·7	5·1	4·7
	(0·31)	(0·33)	(0·13)	(0·11)
5	47·0	19·7	3·3	3·1
	(0·19)	(0·85)	(0·07)	(0·12)

* Numbers in parentheses indicate S.E. of mean.

The mean values for height, half-duration, and the maximum rates of rise and decay, of spikes recorded after 20 min exposure to 20, 10 and 5 mM-NaCl, are shown in Table 2. In all these concentrations activity was maintained at normal frequencies for the longest periods studied (100 min). In 20 mM-Na the maximum rate of rise was only slightly reduced and was probably within normal limits (compare Fig. 4). The maximum rate of decay showed a larger decrease. In 10 and 5 mM-Na both rates were more markedly reduced and the half-duration was further increased. Fig. 15 shows some typical spikes recorded after prolonged exposure to these very low Na concentrations. The time base was adjusted in each experiment so that the errors involved in the graphical determination of the maximum rates of rise and decay were comparable.

If the muscle was exposed to 2 mM-NaCl, the discharge was not usually maintained for periods exceeding 35 min. In the experiment illustrated in Fig. 16, spikes having a normal amplitude were still recorded 15 min after changing over although the rates of rise and decay were reduced. During the next 20 min the height fell to about 45 mV and the rates of rise and decay

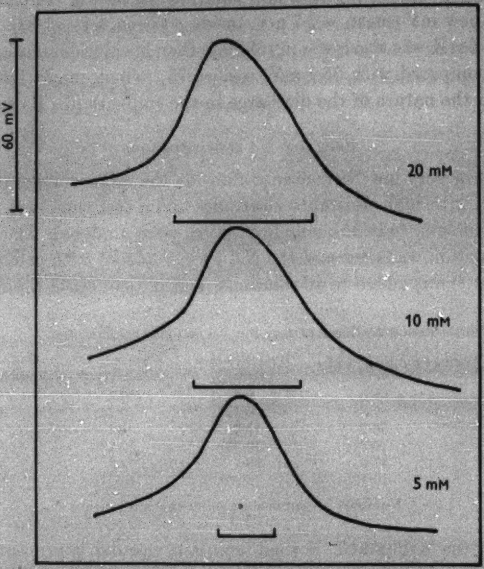

Fig. 15. Examples of spikes in 20, 10 and 5 mM NaCl. Spikes traced from records (swept time base) from three different preparations. Time calibration (horizontal bar) 10 msec.

continued to decrease. At about 30 min the configuration changed abruptly and only slow fluctuations could be seen. The resting potential fell somewhat and after 40 min all activity ceased. Fig. 16 also shows some records from this experiment.

In another experiment the muscle was exposed to 10 mM-NaCl for over 100 min and then the solution was changed to one having zero Na. The spikes changed rapidly to slow fluctuations and after 13 min all activity was abolished. After 20 min exposure to the zero-Na solution, the 10 mM-Na solution was reintroduced; 15 min later activity returned and spikes of up to 60 mV were recorded.

Variation in $CaCl_2$ concentration

If the $CaCl_2$ concentration was reduced to 0·25 normal there was some increase in the activity of the taenia immediately after changing over, but this was not maintained. Records taken after 15 min exposure were indistinguishable from normal.

If the muscle was placed in a solution containing no $CaCl_2$, or one containing 0·05 normal concentration, the initial increase in activity was more pronounced.

Spike frequency rose immediately after changing over, reaching a maximum of about twice the normal rate. The frequency remained high for 30–40 min, after which it began to show violent fluctuations; these continued throughout the exposure period. The mean frequency fell a little, but after 60–70 min it was often higher than during the initial control period. An increase in tension was recorded during the initial increase in frequency but this was often rather small. After about 20 min the tension began to fall in spite of the relatively high frequency of spikes.

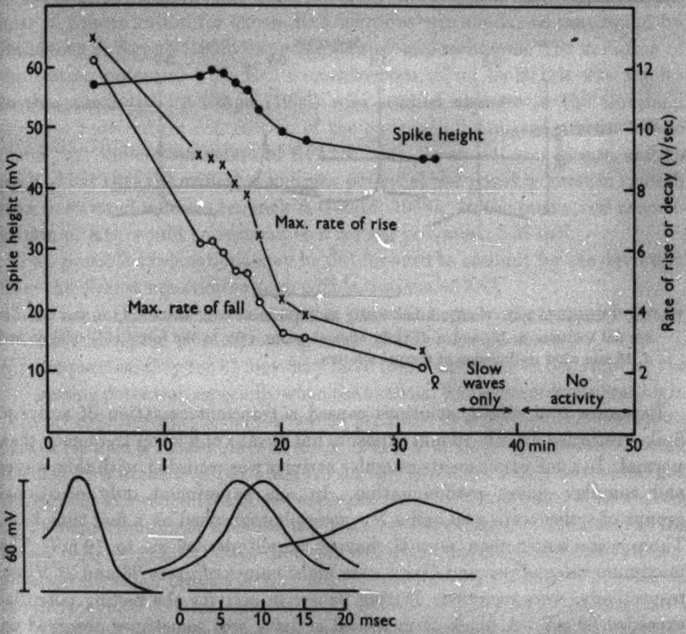

Fig. 16. Changes in spike height, ●, and the maximum rates of rise, ×, and decay, ○, from an experiment with 2 mM-NaCl. The spikes were recorded at 0, 17, 19 and 30 min after changing over. The low Na solution was introduced at 0 min.

Fig. 17 shows the changes in spike configuration which occurred during exposure to Ca-free solution. Record (*b*) was taken 15 min after changing over; the spike height was reduced. Observation on a faster time base showed that the duration was increased owing to a decrease in the rate of both rising and falling phases. Record (*c*) was taken after 45 min exposure. At this time the tension had fallen below normal and the spike frequency, although still high, was fluctuating rapidly. Although it was possible to obtain sharp impalements in Ca-free solution in every case, the initially established resting potential fell rapidly and it seemed that the tip of the electrode could not 'seal in'. A few 'spikes', slightly larger than that shown in (*c*) were seen immediately on impalements, but they did not exceed 40 mV and their configuration was similar to those of (*c*); they never showed an overshoot. During prolonged exposure the 'spikes' in many fibres were not correlated with fluctuations in the tension recorded from the whole taenia. This suggested that the discharges may have been asynchronous in different fibres.

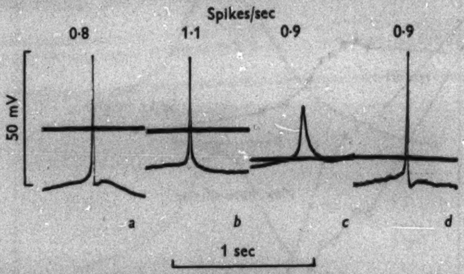

Fig. 17. Changes in spike configuration during an experiment with zero $CaCl_2$; *a*, was taken in normal solution, *b*, 15, and *c*, 45 min after changing over to the zero $CaCl_2$ solution and *d*, 15 min after readmission of normal solution.

Exposure to $4\times CaCl_2$ solutions caused a transient cessation of activity. Spikes returned after 5–10 min exposure but always at a lower frequency than normal. In some experiments irregular activity was recorded, with slow waves and complex spikes predominating. In one experiment only occasional groups of spikes were seen and a few were photographed on a fast time base. Those were larger than normal, having amplitudes of up to 76 mV. The maximum rates of rise and decay were high; values of up to 23 and 21 V/sec, respectively, were recorded. During pauses in activity the resting potential exceeded 60 mV. A phase of increased activity was sometimes observed on re-admission of the normal solution.

DISCUSSION

Resting potential

The resting potential (or, more correctly, the maximum polarization) of the guinea-pig taenia coli was low compared with that of many striated muscles. The mean value during activity was similar to that reported by Bülbring (1954), and to that of other tissues showing autorhythmicity; the resting potential of cardiac pace-maker, for example, was found to be about 55 mV (Hutter & Trautwein, 1956, West, 1955). The resting potential in the taenia, in the absence of the spike discharge, was similar to that of other smooth muscles which were not spontaneously active, such as the sphincter pupillae (Bülbring & Hooton, 1954) and salamander stomach (Greven, 1953). In the taenia these low values may be partly due to the low value of the K equilibrium potential, which is equal to 82 mV (calculated from Born & Bülbring's unpublished results).

The relation between the resting potential and the logarithm of the extracellular K concentration for excess KCl solutions was similar to that found by Burnstock & Straub (1958) using the sucrose-gap technique. The variation of the resting potential with KCl concentrations of up to 30 mM was similar to that predicted by Csapo (1956) who studied changes in the threshold stimulus required for contraction of the oestrogen-dominated uterus. Csapo found that the concentration of KCl for 'zero threshold' was 30 mM, and he pointed out that the critical membrane potential was possibly lower in smooth than in striated muscle (Jenerick & Gerard, 1953). In the taenia this concentration of KCl would correspond to a resting potential of 37 mV.

Two possible explanations may be put forward to account for the deviation from the Nernst equation in high concentrations of KCl.

(i) the intracellular KCl concentration may not have been constant but may have increased with increasing external KCl concentrations;

(ii) the permeability for Cl ions may have been important in determining the resting potential, especially when the external KCl concentration was high.

In my experiments the depolarization produced by very high concentrations of KCl reached a maximum within the first few minutes of exposure. During the next 10 min the depolarization fell slightly to a value which was unchanged throughout the remainder of the exposure period. It was possible, therefore, that a redistribution of ions had taken place. The volume: surface ratio of the taenia cells is small (1·05 μ, Born & Bülbring, unpublished observations). A relatively small uptake of KCl might have led to a significant increase in the intracellular concentration of KCl. Burnstock & Straub (1958) suggested that the Cl ion might have been important in determining the resting potential, since they found a large depolarization on replacing the Cl in normal solution with SO_4. I recorded a much smaller depolarization under these conditions, and this might have been due to a deficiency in ionized Ca. However, I found that the depolarization caused by excess K was larger in sulphate than in chloride solution. In the absence of ionic analyses both factors may be regarded as contributing to the observed relation between the resting potential of the taenia and K concentration.

It was sometimes noted that the resting potential did not return to its normal value at once, on readmission of normal solution, after prolonged exposure to an altered ionic environment. The resting potential in normal solution was low after removal of:

(i) excess KCl concentrations (added to or replacing NaCl);
(ii) isotonic K_2SO_4 (replacing NaCl);

The resting potential was high after removal of:

(i) K-free solutions;
(ii) choline chloride solutions, acetylcholine or histamine (Bülbring, 1957);
(iii) excess NaCl (Holman, 1957*a*).

High resting potentials were therefore associated with conditions where a loss of intracellular KCl had probably occurred during exposure to the test solution (Born & Bülbring, 1956). Low resting potentials were associated with conditions where there had probably been an uptake of KCl (with the possible exception of isotonic K_2SO_4). Hodgkin & Horowicz (1957) have shown, for frog skeletal muscle, that when the Donnan ratio of the K and Cl ions was disturbed (or E_K was not equal to E_{Cl}) the Cl ion could dominate in determining the resting potential. This might also have been the explanation for the results in the taenia. An uptake of KCl might have led to a low E_{Cl} and hence a low resting potential, whereas a loss of KCl might have had the reverse effect.

Spike potentials

The simplest spikes recorded in the taenia coli showed an overshoot which often exceeded 15 mV. This suggested that the ionic movements responsible for the rising phase of the spike might be similar to those of many other excitable tissues which have been described by the ionic hypothesis of Hodgkin & Huxley (1952). This idea was supported by the results of experiments with different $CaCl_2$ concentrations, since the changes in spike configuration in the taenia were comparable with those observed in Purkinje tissue (Weidmann, 1955) and could be explained by changes in the availability of Na carriers (Frankenhaeuser & Hodgkin, 1957). It was found that the spikes in the taenia were abolished in Na-free solution, which was also in accordance with the ionic theory.

However, spikes of normal amplitude were recorded in 20 mM-Na. Though the amplitude and maximum rate of rise fell after prolonged soaking in lower concentrations, spikes recorded after 15 min exposure to 2 mM-NaCl still showed a normal amplitude and 10 mM-NaCl was sufficient to restore the discharge after it had been abolished by Na-free solutions. Somewhat similar observations have been reported by Coraboeuf & Otsuka (1956), who found that there was no correlation between the height of the action potential of the guinea-pig myocardium and the Na concentration of the bathing solution. Thus the action potential height was unaltered in 25% Na, the normal height was maintained for up to 20 min exposure, when there was an abrupt fall in both the resting potential and the action potential height which led to inexcitability.

The behaviour of the taenia coli in very low Na solutions may be taken as evidence that the origin of the spikes is basically different from that in most excitable tissues. Until further evidence is available, however, it seems preferable to regard the membrane of the taenia as having only a limited number of Na carriers and to keep in mind the possibility that some other ion of unknown identity may have been able to replace Na ions on the carrier sites when the Na concentration was very low. The spikes in a normal Na concentration showed a relatively slow rate of rise which was consistent with the idea of a limited availability of Na carriers due perhaps to the low resting potential. Alternatively, the Na carrier mechanism might have been poorly developed in this muscle, which is probably not required to propagate spikes over long distances. A similar explanation was put forward by Burgen & Terroux (1953) to account for the slow rate of rise of the action potential in cat auricle.

The observation that the discharge in the taenia was maintained indefinitely by 5 mM-Na suggested that some other ion might be involved. The intracellular concentration of ionized Ca in smooth muscle is unknown. Hodgkin & Keynes (1957) concluded that almost all the intracellular Ca in squid axoplasm was in an un-ionized state; they also found that the Ca influx was increased during activity. Alternatively, it may be that in the taenia coli choline ions were able to replace Na ions to a limited extent. This possibility has been discussed by Hagiwara & Watanabe (1955), as an explanation for the relation between the height of the action potential in skeletal muscle and the logarithm of the Na concentration, when certain quaternary ammonium ions were used to replace Na. The observation that the spikes in the taenia were abolished in Na-free solution and the lack of any gross changes in configuration in choline solutions make it unlikely that the origin of the spikes in the taenia was similar to that in certain crustacean muscles (Fatt & Katz, 1953).

Spike frequency and tension

If the resting potential was within normal limits a correlation between spike frequency and the resting potential was seen in most but not all of the taenia fibres (Bülbring, 1955). If the resting potential was reduced by, for example, excess KCl, a much closer correlation was seen in all the fibres. In general the changes in spike frequency resulting from changes in ionic environment were associated with changes in resting potential. The initiation of the spike discharges was independent of the concentration of Na and Cl over a wide range. It seems unlikely, therefore, that the ionic movements responsible for the pace-maker activity involved either of these ions.

Throughout these experiments the tension maintained by the taenia was related to the spike frequency, unless the spike configuration became highly abnormal or the activity very irregular. In solutions containing 0·05 normal concentration or no $CaCl_2$ the low tension was probably due to asynchronization of the activities in different fibres. It is interesting that the peaked negative waves, recorded in excess KCl solutions when the resting potential was about 30 mV, were able to maintain a relatively high tension. When these potentials changed to a simple oscillation, as a result of a further depolarization, the tension always fell.

In general the changes in tension observed in the taenia were similar to those reported previously for intestinal muscle. An initial contraction was seen immediately on exposure to KCl-free solution, in agreement with Magee & Reid (1927). The changes in tension in excess KCl solution were similar to those reported by Ambache (1946), Magee & Reid (1927), and Vogt (1943). There was no evidence from the present experiments for an initial contraction on exposure to solutions containing excess $CaCl_2$ (Magee & Reid, 1927). The immediate effect of $4 \times CaCl_2$ solutions on the taenia coli was quite the reverse; the transient relaxation which occurred was very similar to that caused by small doses of adrenaline (Bülbring, 1957).

No evidence was obtained from these experiments for any mechanism other than the spike discharge which could influence the tension of the muscle. The relation between the membrane potential and the activation of the contractile mechanism is still a matter for speculation, even in skeletal muscle (Vaughan Williams, 1954). In the taenia under normal conditions the spike appears to be the initial link in the chain of events connecting excitation with contraction.

SUMMARY

1. Transmembrane potentials from the isolated taenia coli of the guinea-pig were recorded during spontaneous activity with relatively high-resistance capillary micro-electrodes.

2. The mean 'resting' potential (maximum polarization) was 51·5 mV during a spontaneous discharge but values of up to 70 mV were recorded during pauses in activity.

3. The simplest spikes (spontaneous) had a mean amplitude of 59·3 mV, with an overshoot of about 10 mV and a mean duration (at half the total amplitude) of 6·8 msec; the mean values for the maximum rates of depolarization and repolarization were 13·1 and 13·2 V/sec respectively. The spikes showed a variable degree of pre-potential and often a pronounced positive phase.

4. The relation between the resting potential and the logarithm of the K concentration of the bathing solution was linear for K concentrations exceeding 30 mM, with a slope of 33 mV per tenfold change in K concentration (solid KCl added to normal solution).

5. Spike configuration was unaffected by twice the normal concentration of KCl (10 mM). In higher concentrations the height of the spike fell though the 'overshoot' was only reduced by concentrations exceeding 20 mM and abolished by concentrations exceeding 30 mM.

6. K-free solutions caused an initial depolarization and an increase in activity, although all spikes were abolished and the resting potential increased during prolonged exposure.

7. Recovery after prolonged exposure to solutions containing zero or a great excess of KCl was slow: recovery from exposure to low-KCl solutions was associated with a high resting potential and a cessation of the spike discharge, while recovery from high-KCl solutions was associated with a low resting potential and an increase in the frequency of the discharge.

8. The configuration of the spikes was studied in solutions containing very low concentrations of Na ions (20 mM and less). Spike height was normal in 20 mM-Na but fell together with the maximum rates of rise and decay, when the Na concentration was further reduced. The discharge continued indefinitely in 5 mM-Na but was abolished in 2 mM-Na. 10 mM-Na was sufficient to restore the spikes after they had been abolished by Na-free solutions.

9. Solutions containing 0·05 normal concentration, or zero, $CaCl_2$ caused an initial excitation. Although the spike frequency remained high throughout the exposure period the activity was asynchronous and the tension fell. Excess $CaCl_2$ (10 mM) caused a transient cessation of the discharge. Spikes returned during prolonged exposure and many of these showed an increased amplitude.

10. These results are discussed in relation to the ionic hypothesis of Hodgkin & Huxley (1952).

I should like to thank Dr Edith Bülbring who suggested and supervised this work, and also Professor J. H. Burn for his generous hospitality. I am most grateful to Mr O. B. Saxby and Mr Derek Groves for their continued help with the apparatus.

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SPINAL TRACTS AND SUPRASPINAL CENTRES INFLUENCING VISCEROMOTOR AND ALLIED REFLEXES IN CATS*

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Stimulating the abdominal viscera may evoke movements of the limbs and trunk muscles (Miller, 1924; Lewis & Kellgren, 1939; Downman & McSwiney, 1946; von Euler & Sjöstrand, 1947). These visceromotor reflexes may be recorded as compact reflex volleys in the intercostal and lumbar nerves following single-shock stimulation of the central cut end of a splanchnic nerve (Downman, 1955). Using the single-volley analysis it has been shown that the splanchnic-to-intercostal, and also the allied intercostal-to-intercostal, reflexes have different properties in the decerebrate and the spinal cat (Downman, 1955). In the decerebrate these polysynaptic reflexes are smaller, have limited irradiation along the cord, but mutually facilitate each other. Transecting the cervical cord causes the responses to become at once larger and to irradiate far along the cord, but to inhibit each other. In this investigation an attempt has been made to define the spinal paths and brain-stem mechanisms concerned in maintaining the decerebrate–spinal differences in the thoracic arcs. Basically the method has been to determine which tracts must be cut to cause increase in size of the reflexes (release) and change from mutual facilitation to inhibition in a two-volley test (change-over).

METHODS

Adult cats were decerebrated under ether anaesthesia after preliminary tracheotomy and bilateral ligature of the common carotid arteries. The cerebrum was removed piecemeal through a trephine opening in the skull, the spatula being slid downwards on the tentorium cerebelli to cut the brain stem at the intercollicular level. Temporary manual compression of the vertebral arteries in the neck controlled the bleeding from the brain stem. The greater splanchnic nerve on the left side was exposed retroperitoneally and cut distal to the coeliac ganglion. Three of the lower intercostal nerves were dissected free over a length of about 3 cm lateral to the posterior angle of the rib, cutting small side branches but leaving the dorsal ramus intact. The ventral ramus was cut distally in its freed length, the crushed central end being later suspended by a small cotton loop on a stainless steel wire recording electrode. After these operations the animal was usually rested 2–3 hr to excrete the ether. The splanchnic and the upper and the lower of the intercostal nerves could be stimulated with single shocks, delivered from a square pulse generator driven by the oscilloscope time base. Resultant reflex activity was recorded off the intermediate intercostal nerve, using a conventional RC-coupled amplifier and oscilloscope.

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* This report includes work submitted by A. Hussain in part requirements for the degree of Ph.D. in the University of London.

For more extensive exposures of the remaining brain stem, and for removal of the cerebellum, the cranial bone was removed with a dental drill and bone nibblers. Bleeding was controlled by plugging the bone edges with a kaolin paste, and by cautious diathermy. Retrograde air embolism into the bone sinuses was avoided by keeping the head low. Removal of nervous tissue was done with a sucker. Cuts into the brain stem were made with a sucker or with a fine scalpel. During recording, the cats were paralysed with tubocurarine up to 1 mg/kg or decamethonium iodide up to 100 μg/kg, repeated intravenously or intramuscularly as needed, and were given positive-pressure artificial ventilation.

Partial and complete transections of the spinal cord were made with a fine knife after exposing the cord by laminectomy and opening the dura, usually at C 2–3 level. Usually a partial lesion was followed by complete transection about 1 cm more caudally, to compare the fully spinal animal. The extent of the cuts into the spinal cord, and of other brain-stem lesions, was seen on serial sections of formol-fixed material, stained by the method of Loyez (1910) or of Weil (1946). The full extent of the lesions was subsequently mapped by superimposing camera lucida drawings of the sections. Particular attention was paid to the extent of haemorrhage.

RESULTS

Spinal cord sections

Fig. 1 is an example of a cut across the dorsal half of the spinal cord, severing the dorsal and dorso-lateral columns. In this and subsequent figures the size of the response has been measured as the peak height of the volley, from enlargements of the photographic record. The conditioned test response is then expressed as a percentage of the height of the test response alone. In previous work (Downman, 1955) the area of recorded response was used as the best index of magnitude of the reflex discharge. In this investigation peak height was taken to be an adequate index since the differences brought about by the cord lesions were so conspicuous that the method of assessment did not affect the conclusions. It may be noted that the dorsal root reflex makes no significant contribution to the recorded reflex volley (Downman, 1955). In Fig. 1, before the cord section, i.e. in the decerebrated cat, a submaximal reflex volley evoked in 11th intercostal nerve on stimulating 12th intercostal nerve was of a size represented by the vertical line, ●. A conditioning maximum reflex response of splanchnic origin facilitated a later testing submaximal response of intercostal origin, shown by the graph ●. After making the cut into the cord both splanchnic- and intercostally-evoked volleys increased in size; the height of the intercostally-evoked response is indicated by the line ○. Now the two-volley test curve followed the course indicated by the graph ○. Later complete section of the cord caused no more release, height of intercostally-evoked response (shown by the line *Sp*) being unchanged, and the two-volley test curve was also unchanged. It is concluded, from this result, that full release and change-over follows section of some tracts in the dorsal half of the cord. Since the picture was not altered by subsequently cutting the ventral half of the cord also, it is concluded that the tracts therein are not themselves actively causing the changes in cord activity in the absence of the dorsal paths.

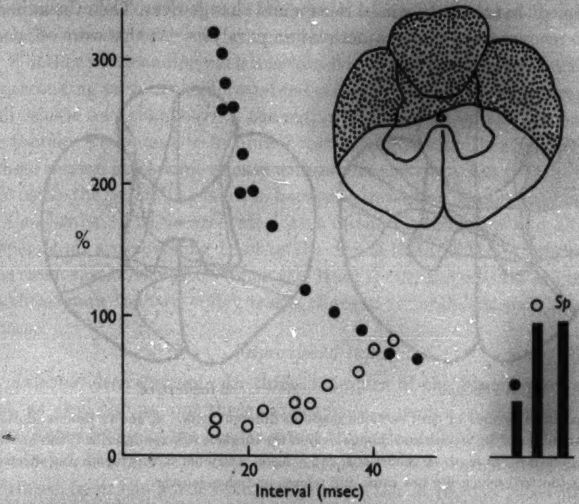
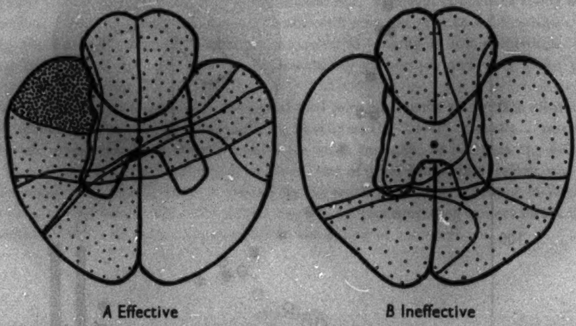


Fig. 1. Dorsal hemisection of spinal cord, shown dotted, in C6 segment in decerebrated cat. In the graph size of a conditioned test intercostal-12-to-intercostal-11 submaximal reflex volley is expressed as a percentage of its unconditioned size. The conditioning volley was a maximal centripetal volley in the ipsilateral splanchnic nerve at the volley intervals shown as abscissa. In the decerebrate before hemisection, ●, conditioning splanchnic volley increasingly facilitates test volley at shorter intervals. After hemisection, ○, conditioning volley inhibits test volley over same time course. Inset right, shows height in arbitrary units of test response alone, in the decerebrate, ●; after hemisection, ○; after later complete spinal transection, *Sp*. Strength of single-shock stimuli unchanged throughout.

By making various cuts in the cord it was found that the results could be grouped into those cuts which caused change-over and those which did not. The former also caused full release of the reflexes of both intercostal and splanchnic origin. The effective cut had to include the dorsal third of the lateral funiculus (Fig. 2) of the same side. In contrast, cutting other regions of the cord caused no change-over (Fig. 2). Cuts into any part of the cord caused some release of the reflexes, but not more than about 30% of the full release found on subsequent complete transection.

Relation to decerebrate rigidity. In two cats the reflex discharges were recorded before and after a cut into the dorsal third of the ipsilateral dorsolateral funiculus, with the animal paralysed with decamethonium iodide. These tests confirmed that the cuts caused release and change-over. Then the animals were left to recover from the decamethonium paralysis. As this wore off, good decerebrate rigidity returned. The spinal pathways which maintain the decerebrate status of the visceromotor and allied reflexes are not the same as those maintaining decerebrate rigidity.


A Effective

B Ineffective

Fig. 2. Composite diagrams of cord sections made in different cats. *A*, shows lesions (dotted) which were followed by release and change-over of the thoracic reflexes described; the heavily dotted region is common to all the lesions, and a lesion restricted to this region was effective. *B*, lesions (dotted) which did not cause full release and change-over.

Ascending or descending pathway? The effective cuts lie in a region of the cord containing both ascending and descending tracts. It has been noted before (Downman, 1955) that transecting the cord in mid-thoracic region does not cause any release or change-over in the headward segments, even in the next segment above the cut. Hence the results cannot be ascribed to loss of ascending impulses originating in levels of the cord below the segment under investigation, such as is responsible for the Schiff–Sherrington phenomenon (Ruch, 1936). Combining total transection of the cord with division on the chest wall of all the ipsilateral intercostal nerves lying rostral does not alter these reflex arcs; loss of the major part of the ipsilateral input from other levels does not seem to be a critical factor. Finally, making a cut into the ipsilateral dorsolateral column in the segment above that used in recording causes release and change-over as effectively as a lesion made higher up. If the inhibition depends on a tonically active ascending path, this path would have to originate in the segment itself. In other experiments it was found that cutting the dorsal roots of the recording intercostal nerve did not alter the responses in decerebrate cats. These findings do not support the hypothesis that the effective cord lesions break an ascending path which begins in ipsilateral dorsal roots below, within, or above the segment tested. The findings do not entirely disprove the possibility of a localized ascending path which is activated by general cord activity maintained by other uncut inputs.

Whether an ascending path is involved or not, it is still necessary to postulate a descending path in the dorsolateral column. Since a cut in this region causes full release and change-over, the majority, if not all, of the descending fibres lie therein. There may be a thinner scatter of fibres in other regions of the cord whose section gives some release without change-over. The present results cannot settle the point. The relation between release and change-over is unknown.

Conclusion. The decerebrate status of the visceromotor and allied reflexes depends on a descending tract in the dorsal third of the lateral funiculus of the same side of the cord. When this tract is cut, the reflexes are released and facilitation in the two-volley test is changed to inhibition over the same time course.

Supraspinal influences

Anaemic decerebration. In three examples of the Sherrington decerebrate cat, as used throughout these experiments, anaemia of the upper brain stem was caused by tying the basilar artery through a hole drilled in the basis cranii (Pollock & Davis, 1923). This caused no release nor change-over. The head was perfused from the aorta with 10% indian ink in reconstituted dried plasma (M. J. Blunt, personal communication). After immersion during the night in 10% formol-saline, the cerebellum and brain stem were removed and cleared by Spalteholz method (Cowdry, 1948). Thick frozen sections were cut in the sagittal plane. It was found that the medulla and lower pons and lower half of the cerebellum retained a blood supply. The centres above the mid pons and the anterior lobe of the cerebellum are not essential in sustaining the thoracic arcs.

Cerebellar lesions. In Sherrington-decerebrate cats the whole dorsal surface of the cerebellum was exposed by removing the overlying bone and tentorium. The size of the reflexes and the form of the facilitation curve in the two-volley test were recorded. Sucking away the anterior lobe of the cerebellum caused no change in the responses. Subsequent removal of the whole cerebellum was also without effect (Fig. 4). These findings confirm and extend the results of anaemic decerebration, and show that loss of cerebellar function was not a critical factor.

Vestibular nuclei. Fulton, Liddell & Rioch (1930*a*, *b*) have related the vestibular nuclei to decerebrate rigidity in the cat. Although the consequences of spinal tractotomies did not suggest that vestibulospinal paths are concerned in controlling the visceromotor arcs, it was considered advisable to test the effects of vestibular nuclear destructions. This was done in cats in which the floor of the medulla had been exposed by complete removal of the cerebellum, as described above. Dividing both VIII nerves intracranially caused no release or change-over. Similarly, removing the vestibular nuclei with a sucker, either ipsilateral or bilateral, caused no change (Fig. 3). Subsequent histological examination showed that the destruction encompassed the main

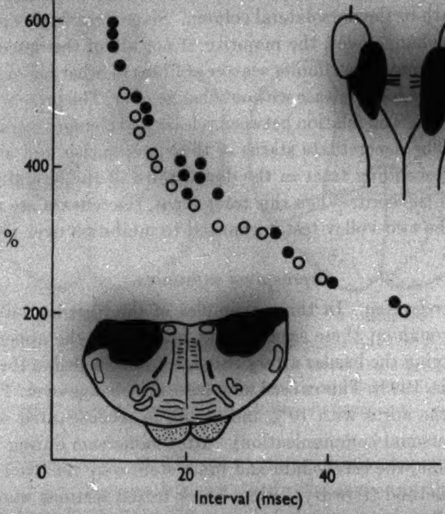


Fig. 3. Graph shows facilitation of reflex motor volley in 11th intercostal nerve, evoked by centripetal volley in 12th intercostal nerve, by preceding centripetal volley in splanchnic nerve. Decerebrate cat, cerebellum removed, ●. After extensive bilateral destruction of vestibular nuclear region, shown in inset diagrams of medulla from above and in section, ○. Height of conditioned test response plotted as percentage of unconditioned response. Submaximal single shock stimulation of nerves. Abscissa shows volley intervals in msec.

bulk of the vestibular nuclei, including Deiters' nuclei. From comparison with published maps of the cat brain stem (Winkler & Potter, 1914) it is estimated that the greater part, probably 90%, of the vestibular nuclei was destroyed. These findings confirm that the vestibular nuclei are not responsible for the decerebrate status of the visceromotor and allied reflexes.

Cuts into the pons and medulla. Deep cuts were made into the medulla after exposing the floor of the medulla by removal of the cerebellum (Fig. 4). Deep transverse cuts into the pons cutting across the central mass of reticular substance were without effect. It was found that wide deep cuts had little effect at levels above the obex. Deep cuts into the lower medulla, at the level of the middle of the cuneate tubercles, caused both full release and change-over. More laterally placed lesions, which would have cut the descending paths from the lateral vestibular (Deiters') nuclei, were without effect. These results show that the decerebrate status of the visceromotor and allied arcs depend upon the action of a portion of the medial substance of the medulla.

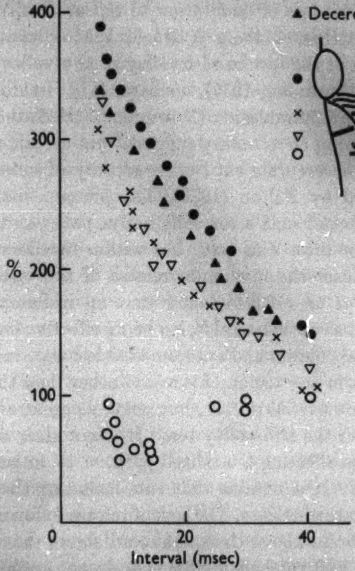


Fig. 4. Graph shows the facilitation of the reflex motor volley in 11th intercostal nerve, evoked by centripetal volley in 12th intercostal nerve, by preceding centripetal volley in splanchnic nerve. Decerebrate cat, ▲; after total removal of cerebellum, ●; deep cut into upper medulla, ×; deep cuts into lateral medulla below vestibular nuclei, ▽. These lesions did not cause release or change-over. Subsequent deep cut into mid-line lower medulla, ○, was effective. Cuts made serially in same preparation in order given. Conditioned test response plotted as percentage of test response alone; submaximal single shock stimulation of nerves.

DISCUSSION

Severing the spinal cord in the decerebrated cat increases some spinal reflex activities, whereas others are depressed so deeply as to merit the term 'spinal shock'. Flexor and extensor arcs show such contrasting change. Sherrington (1910), Sherrington & Sowton (1915), Forbes & Baird (1929), and McCouch, Snape & Stewart (1935) recorded release of the flexor arcs. Flexor reflexes increase in amplitude and have a lower threshold. Other changed activities include increase of the N_1 potential recorded on the dorsum of the cord on cutaneous afferent stimulation (Lindblom & Ottosson, 1952); lowering of threshold of both cord potential and of flexor reflex to that of the nerve (Stewart, Hughes & McCouch, 1940); reappearance of the galvanic skin response (Wang & Brown, 1956); increased size, fall of threshold, and wider irradiation of the splanchnic-to-intercostal and intercostal-to-intercostal reflexes (Downman, 1955); loss of facilitation to repeated afferent volleys in flexor arcs (Liddell, Matthes, Oldberg & Ruch, 1932*b*); change-over from facilitation to inhibition of the second test volley in two-volley testing of the specified thoracic arcs (Downman, 1955); appearance of visceromotor reflexes elicitable by direct visceral stimulation (Downman & McSwiney, 1946). The release of function following chordotomy suggests that certain reflex arcs are kept in restraint in the decerebrate cat by the activity of some region of the brain stem, as proposed by Fulton (1926). The present findings could be explained by the existence of such a tonically active pathway to the thoracic as well as to limb flexor arcs. This path lies within the dorsolateral white column. A cut here causes the maximum release of the visceromotor and allied reflexes. Liddell *et al.* (1932*b*) found that an ipsilateral cord lesion released the flexor reflex, a contralateral lesion being effective only if it crossed the mid line; dorsolateral column and anteromedial lesions were effective. In the present work section in any region of the cord other than the dorsolateral column might cause some partial release, but never as great and not accompanied by change-over in the two-volley test. It is not clear whether this is due to spreading damage affecting a localized path, or to cutting other widespread descending fibres. It is notable that cuts including the anteromedial columns did not cause marked release. The lack of release following destruction of various parts of the brain above the spinal cord shows that corticospinal, rubrospinal, tectospinal and cerebellar influences do not sustain the decerebrate–spinal differences. The active centres lie within the medial region of the medulla.

Liddell and his colleagues (Ballif, Fulton & Liddell, 1925; Fulton *et al.* 1930*a*, *b*; Liddell, 1934, 1936) have shown that chordotomy changes extensor reflexes. Threshold, duration and ease of inhibition increase. This depends on section of the anteromedial cord fibres. Destruction of ipsilateral vestibular nuclei, especially Deiters' nucleus, caused loss of rigidity and a spinal-type knee jerk. The work showed that the vestibular nuclei, through their vestibulospinal paths, are a main but not sole factor in maintaining rigidity and the decerebrate–spinal differences in extensor arcs. Our own tests show that the vestibulospinal tracts and vestibular nuclei are not of importance in imposing the decerebrate–spinal differences in the specified thoracic arcs. These arcs again behave more like flexor than extensor arcs. Cutting N. VIII in the acute decerebrate caused no change in thoracic arcs. In contrast, Liddell, Matthes, Oldberg & Ruch (1932*a*) reported that in the cat decerebrated within a week of N. VIII section there was some release of ipsilateral flexors, but more release of the contralateral; flexor threshold was lower after chordotomy than after N. VIII section.

In the specific thoracic reflex arcs the decerebrate–spinal differences are maintained by structures in the medial upper medulla. This conclusion may be related to Torvik & Brodal's report (1957) on the origin of reticulo-spinal paths from the medial medulla and pons, but one notes that the effective spinal cord lesion appears to be more dorsal and compact than the main distribution of the descending medullary fibres of Torvik & Brodal. Also the pathway related to the thoracic arcs has ipsilateral origin, whereas the reticulo-spinal fibres of medullary origin have bilateral origin (Torvik & Brodal, 1957). This dorsolateral lesion would, however, probably cut the lateral reticulo-spinal pathway described by Papez (1926).

The fibres whose section causes release of the reflexes cannot be distinguished from those whose section causes the change-over from facilitation to inhibition in the two-volley test. If they prove to be the same, then the change-over may be a consequence of release from an inhibition somewhere in the arc. Liddell *et al.* (1932*b*) have also reported loss of facilitation of a flexor reflex with repeated stimuli after chordotomy in the decerebrate cat.

That the release and change-over of the reflexes is due to dividing an ascending, rather than a descending, pathway merits consideration. However, it has been described above that cutting the cord does not cause cephalad release, in spite of the section of a large number of ascending paths. Making the dorsolateral cord lesion one or two segments above the segment of recording is as effective as a higher lesion. Hence, if an ascending path is involved it would have to be driven by the thoracic segment under investigation. Cutting the dorsal roots of this segment does not alter the reflexes. The evidence is against an ascending pathway from the cord itself being a major factor in sustaining the tonic inhibition. In any case, it is still necessary to postulate a descending pathway from the medulla. Since cutting regions of the cord other than the ipsilateral dorsolateral segment is much less effective than section of this segment alone, it is clear that the descending path is most concentrated in, if not limited to, this region. This path can be cut without altering the decerebrate rigidity of the limbs.

SUMMARY

1. Splanchnic-to-intercostal and intercostal-to-intercostal polysynaptic reflexes have been recorded in decerebrated cats.

2. Spinal cord transection causes release of the reflexes from a tonic inhibition. This is accompanied by change-over from facilitation to inhibition in two-volley tests.

3. The tonic inhibitory path lies in the ipsilateral dorsolateral segment of the cord.

4. The inhibition is sustained by the medial medulla.

5. The decerebrate status of these thoracic arcs persists after anaemic destruction of remaining brain stem above the pons, including the red nucleus; after partial or total removal of cerebellum; after section of N. VIII unilaterally or bilaterally; after destruction of vestibular nuclei unilaterally or bilaterally; and after cutting other segments of the cord, including the vestibulospinal paths.

6. A dorsolateral column lesion in the cord causes release and change-over of these reflexes without change of decerebrate rigidity.

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CARDIOVASCULAR RESPONSES TO ELECTRICAL STIMULATION OF THE BRAIN STEM

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The significant role of the medulla oblongata in the regulation of cardiovascular function was first clearly defined by the classical transection studies of Dittmar (1870, 1873) and Owsjannikow (1871). Beginning with localization of pressor and depressor points in the floor of the fourth ventricle by Ranson & Billingsley (1916), many workers have contributed to current concepts of a diffuse distribution within the lateral reticular formation (pressor) and the caudal part of the medial reticular formation (depressor) of a network known as the vasomotor centre (Wang & Ranson, 1939; Monnier, 1939; Alexander, 1946; Bach, 1952). Chen, Lim, Wang & Yi (1936, 1937) proposed that this reactive region is a general sympathetic area inducing extensive sympathetic activation. Bach (1952) reported that stimulation of points within the diffuse network of the vasomotor centre yields simultaneous cardiovascular, respiratory and somatic (knee jerk) responses. Much of this work suggests that the medullary reticular formation in which the vasomotor centre is enmeshed is a relatively non-specific system. However, recent neuro-anatomical studies (Scheibel & Scheibel, 1958), which reveal the extensive synaptic connexions of a single reticular neurone, and which permit estimation of the large number of such neurones activated by commonly used stimulation procedures, suggest that such an interpretation of functional activity within the reticular formation should be made with caution.

Recent writers (Cotten, 1953; Randall & Rohse, 1956; Anzola & Rushmer, 1956) have re-emphasized the significance of the sympathetic outflow to the heart. On the basis of these studies, it appears that the more important functional effect of sympathetic activation of the heart is augmentation of myocardial contraction. When the left stellate ganglion is stimulated, cardioaugmentor responses with large increases in systolic pressure are recorded, while diastolic pressure and heart rate remain relatively unchanged. Similar responses have been reported during stimulation of the brain stem reticular formation (Peiss, Middo, Randall & Jones, 1956; Peiss, 1956).

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In reviewing the current status of bulbar regulation of cardiovascular function in the light of these studies, several important facts appear. There has been no description of cardio-augmentor responses following brain stem stimulation. With few exceptions, workers in this field have measured pressor or depressor activity with no attempt to analyse these responses. Moreover, the majority of these workers, having used inadequate recording techniques, could not analyse pulse pressure changes so as to interpret possible cardio-augmentor effects. In several instances (Alexander, 1946; Pitts, Larrabee & Bronk, 1941) electrical activity in the inferior cardiac nerve has been recorded as a measure of cardio-accelerator discharge. However, optical pressure pulses simultaneously measured often show no change in either heart rate or pulse pressure. The conclusion seems warranted, therefore, that increased electrical discharge in the inferior cardiac nerve is not necessarily a valid criterion of cardio-acceleration or cardio-augmentation. Finally, there is little direct evidence of a cardio-accelerator centre at the medullary level. This latter point will be treated in detail in a separate communication. One must conclude, therefore, that the concept of cardiac regulating centres in the diffuse network of the vasomotor area is largely based on inference from reflex studies.

It is the purpose of this report to describe cardio-augmentor responses to stimulation of the brain stem, to demonstrate activation of specific types of cardiovascular responses, and to assess the relation of these findings to cardiovascular reflexes and specificity of the reticular formation.

METHODS

Fourteen dogs and fifty-three cats were used. Various anaesthetic agents were used, but the majority of experiments were performed on animals under sodium pentobarbital or alpha-chloralose anaesthesia. There were no significant differences in response, regardless of the anaesthetic employed. Arterial blood pressure was recorded from a carotid artery with a Sanborn electromanometer adapted to drive a Sanborn EKG optical galvanometer. Square-wave stimulation pulses were delivered from an American Electronics Model 104 square-wave generator, which was continuously monitored by an oscilloscope connected across the electrodes. Actual stimulation voltages were read from the oscilloscope. Experiments on dogs were performed by surgical exposure of the floor of the fourth ventricle and manual placing of unipolar electrodes on or just ventral to the floor of the ventricle. All cats were positioned in a Johnson stereotaxic apparatus. In some cases small holes were made in the cranium and electrodes inserted stereotaxically into the areas desired. In other experiments occipital bone was removed, part of the cerebellum aspirated by suction, and electrodes then positioned under stereotaxic control. In the cat experiments electrodes were of the coaxial type, the outer shell (reference electrode) being 22 gauge hypodermic-needle tubing completely insulated except for the terminal portion of the ring. The active electrode consisted of fine stainless steel wire projecting 1 mm beyond the reference electrode and insulated except for the terminal 0·5 mm, which tapered to a dull point. Electrodes were routinely tested for integrity of insulation. Stimulation voltages varied from

2 to 7·5 V. Pulse duration was usually kept constant at 2 msec, with the exceptions noted in the text. Frequency of stimulation was varied from 50 to 100 c/s in the experiments reported here, and the duration of stimulation was 15 or 20 sec. At termination of the experiments the brain stem was fixed with formalin for subsequent histological work-up. In order to limit cardiac responses to those mediated by the sympathetic nervous system, bilateral vagotomy was carried out on all animals.

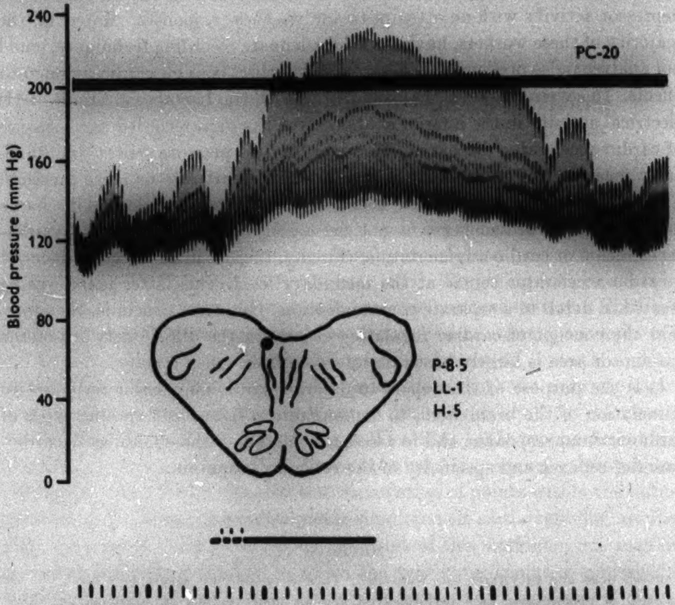

Fig. 1. Cardio-augmentor response elicited by stimulation (3 V, 2·0 msec, 70 c/s) of cat's lower brain stem at breaks in lower horizontal bar. Black dot in cross-section of brain stem indicates site of stimulation in Horsley–Clark co-ordinates. Time marker 1 sec.

RESULTS

Cardio-augmentor responses

Over 100 different points (Horsley–Clark co-ordinates) in the pons and medulla have been stimulated. Owing to the large number of responses obtained, it is impossible to do more than illustrate certain types of response common to a large number of animals. Detailed mapping of reactive points awaits the completion of the histological examination. The significant points to be made from these experiments are illustrated in the figures below. Fig. 1 shows the effect of stimulation in the rostral medulla just ventral to the floor of the fourth ventricle, and 1 mm to the left of mid line. This response is the closest we have come to eliciting a 'pure augmentor' effect, and looks strikingly like those obtained by Randall & Rohse (1956) when the left stellate ganglion was stimulated. Systolic pressure rose from a control level of 150 to 230 mm Hg, while diastolic pressure changed only from 110 to 140 mm Hg. Heart rate remained constant during and after stimulation. The change in pulse pressure strongly suggests that the primary effect of stimulation at this point in the brain stem was activation of neurones which eventually terminate as post-ganglionic fibres on the myocardium, and which induce greatly increased myocardial contraction. There was no evidence of significant vasoconstriction. The latency between the onset of stimulation and the first augmented beat of the heart was less than 4 sec. Since both augmentation and acceleration of the heart beat also occur secondary to adrenal secretion, it is essential to restrict interpretation of direct sympathetic augmentation of the heart to those responses occurring within 6 sec of onset of stimulation. It is quite common to find latencies as short as 2 sec (see below), a value which compares closely with latencies observed during sympathetic trunk stimulation.

Although it is tempting to compare responses to restricted stimulation of the isolated stellate ganglion and a generalized stimulation in the brain stem, the comparison is weakened by the fact that brain stem stimulation may influence vascular function in wide-spread areas of the body. This is due in part to the profuse interconnexions within the reticular formation. Moreover, it is not known whether the point stimulated is on an afferent or an efferent pathway. In order to extend the comparison, the spinal cord was transected at the T4–T5 level in six animals. This procedure eliminates nervous excitation of the adrenal medulla and also eliminates a large fraction of peripheral vasoconstrictor fibres. Although hypotension results from the transection, it was still possible to elicit significant cardio-augmentor responses. Within a few seconds of onset of stimulation augmented pulse pressure was observed. The effect of stimulation was of long duration, a common finding when the stellate ganglion is stimulated. Thus, although the magnitude of the response in a 'transected' animal was always less than in the 'intact' animal, it is clear that significant cardio-augmentation can occur in animals with cord transection at the T4–T5 level.

Adrenal responses

Figure 2 (dog no. 14) indicates a type of response which should not be interpreted as a direct nervous cardio-augmentation. For 18 sec following the onset of stimulation no significant change was observed in either pulse pressure or heart rate. A modest vasoconstriction occurred, resulting in a small rise in arterial pressure. At the end of this period of time a simultaneous increase occurred both in pulse pressure and heart rate. The timing of these events suggests that the long-latency response was due to secretion of adrenaline from the adrenal medulla, with subsequent inotropic and chronotropic effects on the heart, and with a further vasoconstriction of peripheral blood vessels. Responses of this type have been eliminated by acute bilateral adrenalectomy (Peiss, 1957). The late onset of augmentation and/or acceleration is a common occurrence. In most cases it is preceded by a short-latency vasoconstriction, presumably of splanchnic origin. These delayed responses are sometimes attributed to direct sympathetic stimulation with delayed peripheral transmission. It is our experience, however, that brain stem stimulations of direct sympathetic pathways to the heart show latencies as low as 1·8 sec. Responses occurring with latencies approaching the animal's circulation time should be considered to be of adrenal origin unless evidence to the contrary is presented.

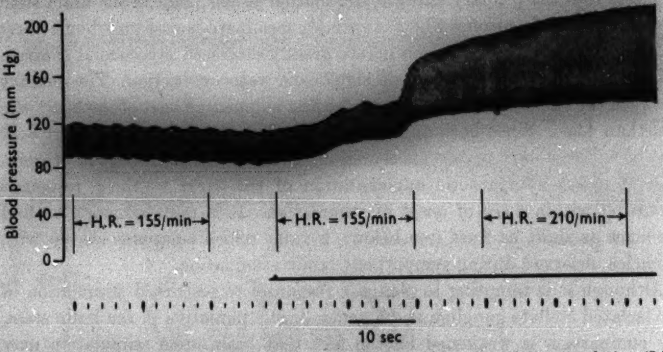

Fig. 2. Delayed augmentation and acceleration of dog's heart elicited by stimulation (3 V, 2 msec, 100 c/s) of brain stem, horizontal bar. See text for discussion.

Mixed responses

Figure 3 is an example of the most common cardiovascular response to stimulation of the dorsal medulla. Within 2·5 sec of the onset of stimulation (3 V, 2 msec, 70 c/s) a tremendous pressor response began, reaching a maximum in about 25 sec. Careful examination of the figure reveals increased pulse pressure (augmentation) on the 6th beat after onset of stimulation. At the peak of the response pulse pressure had increased from a control level of 18 to 62 mm Hg. This is superimposed upon a marked elevation in diastolic pressure, which is interpreted to indicate increased peripheral resistance. It is characteristic that such maximum increases in blood pressure are achieved only when vasoconstriction and cardio-augmentation occur simultaneously. The electrode was then lowered 1 mm ventrally and the response illustrated in Fig. 3 changed to one in which pulse pressure remained relatively constant, while the maximum mean pressure attained was some 50 mm less than that shown in the figure. The vertical dotted lines in Fig. 3 are spaced 10 sec apart; numbers between the lines indicate the number of heart beats in that period. It is clear that no significant increase in heart rate occurred at the time that both vasoconstriction and cardio-augmentation were induced by the stimulus.

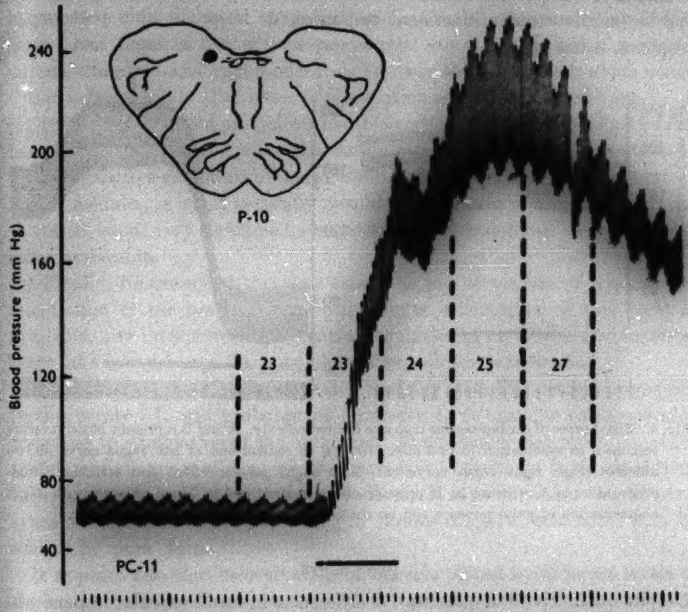

Fig. 3. Mixed cardiovascular response in the cat to stimulation (3 V, 2 msec, 70 c/s) of brain stem, horizontal bar. Note large rise of blood pressure with augmented pulse pressure and lack of significant cardio-acceleration. Broken vertical lines are spaced 10 sec apart; numbers between lines represent number of heart beats/10 sec.

Reflex responses

Figure 4 shows that separation of cardiovascular responses can also occur by reflex stimulation. In parts A and B of this experiment, taken from two different dogs, the central end of the cut left vagus nerve was stimulated at 5 V, 2 msec, 100 c/s. In each case the right vagus nerve had also been cut. Part A shows primarily a modest pressor effect and a most striking cardio-acceleration occurring within a few seconds of the onset of stimulation. The origin and central connexions of the afferents in the left vagus nerve, whose stimulation results in sympathetic cardio-acceleration, are not known. In part B similar central left vagus stimulation in another animal resulted in a prompt large pressor response with slight augmentation of the pulse pressure but with no increase in heart rate. In each animal, moreover, variation of stimulation parameters resulted in marked changes in the type of cardiovascular response. Whether or not specific cutaneous and visceral afferents result in activation of specific cardiovascular functional responses, or in predictable patterns of response, is not known.

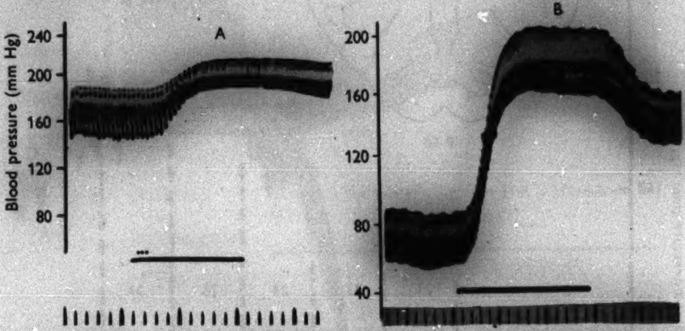

Fig. 4. Mixed types of cardiovascular response elicited reflexly. A and B represent blood pressure responses to stimulation (5 V, 2 msec, 100 c/s) of central end of left vagus nerve in two different dogs; right vagus nerve cut. Note slight pressor effect and striking cardio-acceleration in A, whereas in B primary effect is large rise in blood pressure with slight augmentation of pulse pressure and no change in heart rate. Time marker, 2 sec.

DISCUSSION

Despite qualitative and quantitative differences in cardiovascular responses to electrical stimulation throughout the medulla, certain generalizations are permissible.

(1) The most commonly elicited response is vasoconstriction, either alone or associated with cardiac and adrenal effects. For the present it seems most reasonable to ascribe this to the relatively large number of central fibres or cells representing vasoconstrictor innervation of the blood vessels.

(2) Analysis of pulse pressure changes, correlated with reports of dynamic effects of sympathetic trunk stimulation, indicates that within the network of the vasomotor centre are cells or fibres whose stimulation results in increased force of myocardial contraction. Although commonly associated with simultaneous vasoconstriction, such cardio-augmentor activity is sometimes seen as the only significant result of brain stem stimulation. This response is readily seen in animals with spinal cord transection at the T4–T5 level.

(3) These experiments confirm anew the concept of cells or fibres in the lower brain stem whose activation results in secretion by the adrenal medulla. In addition, they indicate that the common occurrence of adrenal secretion necessitates careful attention to the latency of response to stimulation of the brain stem. Adrenal secretion results in both chronotropic and inotropic changes in cardiac activity. The determination of cardio-acceleration or cardio-augmentation on the basis of sympathetic activation via stimulation of the brain stem requires one of two procedures: (*a*) the preparation must be adrenalectomized and vagotomized, or (*b*) only those responses which occur within a few seconds after onset of stimulation can be considered valid.

(4) In the vagotomized animal cardio-acceleration via sympathetic pathways has never been observed when the dorsal half of the medulla is stimulated. The few positive responses obtained resulted from stimulation of the ventrolateral medulla, in areas generally devoid of significant pressor activity. On the other hand, very significant sympathetic acceleration can be achieved by reflex activation.

(5) Finally, we cannot at this time assess the role of venomotor responses to stimulation of the brain stem. Until definite information on this point is available, any interpretation of central regulation of cardiovascular function must take into account the possibility of venomotor participation.

On the basis of these findings certain promising areas of investigation require further study. It will probably be necessary to re-examine cardiovascular responses to stimulation of the brain stem, using smaller electrodes. In order to differentiate types of response, it is necessary to excite far fewer fibres than is the case with usual stimulation procedures. It is possible that the different types of responses, usually occurring in various combinations, could then be elicited in 'pure' form.

It appears also that re-evaluation of the role played by different levels of the brain stem in cardiovascular regulation is necessary. Our results emphasize important medullary control of myocardial contractility. Some of our unpublished results, as well as those of McQueen, Browne & Walker (1954), indicate that this control is not restricted to the medullary level. Sympathetic regulation of heart rate does not appear to be integrated at the medullary level. A significant number of blood vessels appear to be under control of nervous mechanisms that also have no integrated representation in the medulla (Uvnäs, 1954; Lindgren, 1955). It seems essential to us that the description of cardiovascular activation must now be made in terms of what specific mechanism(s) has been activated, and what level(s) of the central nervous system is involved.

SUMMARY

1. Cardiovascular responses elicited by electrical stimulation of the lower brain stem have been studied in bilaterally vagotomized cats and dogs under sodium pentobarbital and alpha-chloralose anaesthesia.

2. Vasoconstriction is the most common response to such stimulation. Often it is associated with adrenal and/or cardiac effects.

3. Within the area studied are sites whose stimulation results in greatly increased force of myocardial contraction. This is sometimes unaccompanied by vasoconstriction and almost always unaccompanied by direct sympathetic cardio-acceleration. The cardio-augmentor responses can be obtained in animals with spinal cord transection at the T4–T5 level.

4. Adrenal responses generally result in simultaneous inotropic and chronotropic effects on the heart, and are characterized by a long latency between the onset of stimulation and the resulting cardiac effects.

5. The dorsal medulla and pons do not contain cells or fibres whose stimulation results in sympathetic cardio-acceleration. Sympathetic acceleration, however, can be activated by reflex stimulation.

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5-HYDROXYTRYPTAMINE AND HISTAMINE IN MAST CELLS OF THE MOUSE AND RAT

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Tissue mast cells are now known to contain at least three compounds of outstanding physiological and pharmacological interest. Two of these, heparin and histamine, appear to be universally present in the mast cells of higher vertebrates (Jorpes, 1946; Riley & West, 1953). There is thus a positive correlation between the mast cell content of a particular tissue and the amounts of heparin and histamine that can be extracted from it. Very high values for heparin and histamine are found in tissues which are exceptionally rich in mast cells, such as the cleaned capsule of normal ox liver (Riley, 1955), and in certain pathological conditions such as urticaria pigmentosa in man (Riley & West, 1953) and mast cell tumours in dogs (Cass, Riley, West, Head & Stroud, 1954).

The situation with respect to 5-hydroxytryptamine (5-HT) in mast cells is entirely different. Assay of the tissues mentioned above—ox liver capsule, urticaria pigmentosa, dog mastocytomas—reveals only negligible quantities of 5-HT (R. Cass, unpublished data). On the other hand, it does appear that 5-HT may be a normal constituent of the mast cells in the mouse (Sjoerdsma, Waalkes & Weissbach, 1957) and in the rat (Benditt, Wong, Arase & Roeper, 1955).

As is well known, 5-HT is formed in the gut, and is stored mainly in the blood platelets. In addition, Benditt *et al.* (1955) have found a relatively high content of 5-HT in tissue mast cells isolated from the peritoneal cavity of rats: Bhattacharya & Lewis (1956) have likewise shown a positive correlation between the mast cell content of rat skin and its content of 5-HT. However, Parratt & West (1957*a*, *b*) believe that the mast cells may not be the sole site of the 5-HT in rat skin since much of the total 5-HT remains in the epithelial layer after the subcutis with its mast cells has been removed. It therefore seems desirable to seek fresh evidence regarding the association of 5-HT with the mast cells in the mouse and rat, preferably in tissues remote from both gut and surface epithelium.

So far as the mouse is concerned, a mastocytosis can be induced by painting the skin with a carcinogenic hydrocarbon (Cramer & Simpson, 1944). Unfortunately it is then hardly possible to separate completely epidermis from dermis, though the majority of the mast cells are removed in that portion of the dermis which can be scraped off the deep surface of the skin with a scalpel. However, we were able to confirm the presence of 5-HT in the mast cells of the mouse using another tissue which consists entirely of well granulated mast cells free from epithelium. This is the transplantable mouse mastocytoma recently isolated by Dr Jacob Furth of Boston (Furth, Hagen & Hirsch, 1957).

No lesions rich in mast cells comparable with the above are as yet available in the rat. However, Urist & McLean (1957) have recently described a curious mastocytosis in the bones of rats fed on a special diet ('Diet E', Shohl & Wolbach, 1936), which produces a mixed syndrome of rickets and osteitis fibrosa. The effects of a liberator of histamine (compound 48/80) and a liberator of 5-HT (reserpine) were also tested on such animals. We have utilized these various tissues from mice and rats to show that 5-HT as well as histamine is present in the mast cells of the two species.

MATERIALS AND METHODS

Mice

Skin. M.R.C. strain white mice of either sex (average weight, 18 g) received on two successive days a surface application of 0·05 ml. of a 1% (w/v) solution of 9:10-dimethyl-1:2-benzanthracene (L. Light and Co.) in acetone. This was applied to the centre of the back from a tuberculin syringe. Control mice were painted with acetone alone.

Two months later, when the first small papillomas were appearing in the carcinogen-treated areas, the animals of both groups were killed by cervical dislocation and skinned, and the painted areas were excised. A small portion was removed from the centre of every third specimen for histological examination: these samples were spread on pieces of filter paper, fixed in formol-corrosive, embedded in paraffin, cut at 10 μ and stained with 0·1% (w/v) toluidine blue for mast cells.

The dermis was then vigorously scraped off the remaining portions of skin from each group, and the scrapings, containing the mast cells, were pooled, weighed and placed in 20 vol. acetone for extraction of 5-HT. Histamine was not assayed in this material since it is already known that the histamine value rises in proportion to the mast cell content (Riley & West, 1953).

Mast cell tumour. Through the kindness of Dr Jacob Furth we obtained weighed, lyophilized portions of the transplantable mast cell tumour isolated by him from a group of mice which had been subjected to ionizing radiation. Though capable of growing to a considerable size, this tumour is benign and is remarkable in that it is entirely composed of well differentiated mast cells. Samples of the dried, lyophilized tumour were extracted for histamine and 5-HT by the standard methods described below.

Rats

Bones. Weanling rats, Wistar strain of either sex (average weight, 35 g) were fed on Shohl's 'Diet E' (Shohl & Wolbach, 1936) for 6–8 weeks. Control groups of weanlings received rat cake (Rat Cake, Diet No. 36, North Eastern Agricultural Co-operative Society Ltd., Aberdeen). All groups were given water *ad lib.* and at the end of the feeding period the rats were killed by placing them in an anaesthetic box filled with CO_2. The bones of all four limbs were quickly dissected out and scraped free from other tissue. The rats on 'Diet E' had gained but little weight, and even at this stage it was evident that their bones were fragile and so poorly calcified that the marrow was visible through the cortex. The bones of one hind limb from each rat were used for determination of histamine, those of the remaining three limbs for 5-HT. Representative samples of bone from each group were retained for histological study.

Extraction and assay of histamine. Histamine was extracted from the mouse tissues and from the bones of the rachitic rats by the method described by Riley & West (1953) using 10% trichloroacetic acid (TCA), there being insufficient calcium in these bones to interfere with the extraction and assay. However, difficulties were encountered with bones from normal rats, due to the large amount of calcium which was also extracted by the TCA. Bulky precipitates of calcium were thrown down during the later stages of extraction of the TCA with ether, as the pH of the extracts approached neutrality. At the same time histamine was lost, presumably through adsorption on to the precipitate. This difficulty was eventually overcome by precipitating the calcium with conc. H_2SO_4 before extracting the TCA with ether. The final procedure adopted was as follows: the tibia and femur from one hind limb, together weighing about 1 g, were crushed in a mortar and finely ground with sand. The ground bone was added to 2 ml. of 10% TCA in a centrifuge tube and the mortar was washed with a further 1 ml. of TCA. The mixture was shaken thoroughly and immediately centrifuged (2500 rev/min on an MSE Major Centrifuge) for 10 min, after which the supernatant was poured into a fresh tube. In this way the amount of calcium dissolved by the TCA was reduced to a minimum. Parallel extraction of rat ears showed that the whole of the histamine was extracted by the 10–15 min contact of ground tissue with TCA.

Six drops of conc. H_2SO_4 were then added to the TCA supernatant; the tube was cooled in the refrigerator for 10 min, and the precipitated calcium sulphate removed by centrifuging. Thereafter the supernatant was poured into a clean tube and extracted three times with 4 vol. ether as in the original method. If further precipitation of calcium sulphate occurred, the tube was again gently centrifuged (1500 rev/min) after the last shaking with ether, to give a final clear separation of precipitate, aqueous layer and ether. Loss of histamine on these precipitates did not occur, since the supernatant remained highly acid throughout, owing to the presence of excess H_2SO_4. The final extract was then brought to pH 6 with 20% NaOH before being assayed.

As a pilot experiment, bones from twelve normal rats and nine 'Diet E' rats were extracted by this modified method and the results compared with those obtained using the normal method of extraction.

The histamine content of all extracts was determined by assay on the atropinized isolated guinea-pig ileum, using automatic apparatus and the four-point technique. The error of the assays was within ±5%. It was shown that the spasmogen was, in fact, histamine by recording parallel recovery curves for standard histamine and representative extracts after the addition of mepyramine to the organ bath.

Extraction and assay of 5-HT. 5-HT was extracted from all tissues with acetone.

Bones were dissected, cleaned, crushed with forceps and dropped into a previously weighed covered beaker of A.R. acetone, the bones from five or six rats being pooled. The weight of the bones was then calculated by difference, and the acetone made up to 20 vol. The bones were further cut up under the acetone with scissors and allowed to stand for 1 hr, the supernatant being then filtered off and the residue again cut up or ground with sand, and washed with 95% acetone for a second hour. The combined filtrates were evaporated to dryness *in vacuo* and stored overnight at −14° C.

Blood was removed directly from the heart by means of a pipette and collected in graduated centrifuge tubes containing 0·5 ml. of 3·8% sodium citrate. The blood was centrifuged at 800 rev/min for 20 min and the plasma then centrifuged at 3500 rev/min for 10 min. The supernatant plasma was decanted off the platelet 'button' and both were extracted with acetone (4 vol. for plasma, 5 ml. for platelets), the remaining procedure being as for bones. All glassware was siliconed.

Subcutaneous tissue was obtained by skinning the rat and scraping the under surface of the skin with a scalpel. The scrapings were weighed and extracted as before, except that when the extract reached small volume it was shaken with petroleum ether (b.p. 40–60° C) to remove lipids, before being taken to dryness.

All samples were assayed quantitatively for 5-HT on the oestrous rat uterus using de Jalon's fluid as modified by Gaddum, Peart & Vogt (1949), containing atropine, 10^{-7} g/ml. Thereafter a specific inhibitor of 5-HT, lysergic acid diethylamide, 10^{-7} g/lm., was added to the perfusion fluid. This abolished all contractions, except those due to other smooth-muscle-stimulating substances in plasma.

Qualitative confirmation of 5-HT was obtained by paper chromatography. Rachitic bones were extracted in the normal manner, and the dry residue was taken up in 0·5 ml. distilled water. This was centrifuged at 13,000 rev/min (18,238 *g*) to remove the remaining fat globules and other suspended matter. The clear supernatant was applied to Whatman No. 4 filter paper, in three spots, together with appropriate reference markers, each of 2 μg 5-HT. One paper was run as an ascending chromatogram in butanol:acetic acid:water (4:1:5) and developed with Ehrlich's reagent, and the other two in butanol:ammonia (1:1). One of these was developed with Ehrlich's reagent, and the other with Jepson's reagent. In each case the extract yielded a spot corresponding exactly in colour and R_F value to that of the standard 5-HT. With Jepson's reagent a similar fluorescence was observed in both.

Effects of compound 48/80 *and reserpine on rat tissues.* It is well known that compound 48/80 is a highly efficient histamine liberator in the rat, acting mainly on the histamine in the mast cells of the skin (Riley & West, 1955*a*, *b*). Reserpine is likewise capable of removing 5-HT from the blood platelets (Shore, Pletscher, Tomich, Kuntzman & Brodie, 1956). It was thus of interest to determine the effects of these two agents on the raised histamine and 5-HT contents of the bones of the rachitic rats.

Compound 48/80, in graded doses of 0·5–5·0 mg/kg, increasing by 0·5 mg/kg at each injection, was injected subcutaneously twice daily for 5 days into groups of twelve normal and twelve 'Diet E' rats. This is known to remove almost completely the histamine from the skin, and especially the ears, which can be used as an index of the effect (Riley & West, 1955*a*).

Reserpine was given as a single intraperitoneal injection of 1 mg/kg to twelve 'Diet E' rats 16 hr before they were killed. This dose of reserpine displaces the 5-HT from the platelets, thus removing one possible source of error in the localization of 5-HT in the tissues.

RESULTS

Mice

Skin. Histological examination confirmed the well-known effect of a carcinogenic hydrocarbon in augmenting the mast cell population in the dermis of treated mice (Pl. 1, figs. 1, 2). This was accompanied by a substantial increase in the 5-HT content of the precancerous skin, the mean value rising from 0·169 to 5·40 μg/g.

Tumour. Any doubt which might have remained regarding the presence of histamine and 5-HT in the mast cells of the mouse was removed when the mast cell tumour was examined, for this contained no less than 28 μg/g of 5-HT and 1700 μg/g of histamine.

Rats

Bones. Histological confirmation was obtained of the extraordinarily rich mast-cell content of the bones of rats fed for 6–8 weeks on 'Diet E', the increase in mast cells being maximal in the metaphyseal region, slightly away from the epiphyseal plate. An occasional mast cell may be found under the cortex of the metaphysis in the normal rat. However, in the 'Diet E' rats the entire metaphyseal region is studded with mast cells which surround the altered bone trabeculae, as described and illustrated by Urist & McLean (1957) (Pl. 1, figs. 3, 4). Similar changes, though less marked, occur throughout the bone structure generally: a few new mast cells are present in the framework of the medulla and around the vessels which traverse the decalcified cortex. From the histological standpoint no tissue other than the bones is affected, though as mentioned previously animals on 'Diet E' fail to gain weight. The mast cell content of all other organs remains unchanged.

TABLE 1. Comparison of values obtained for histamine content of rat bones when extracted by the normal method and when the calcium is first precipitated with H_2SO_4

Treatment	No. of rats	Mean histamine (μg/g)	S.E.	*P*
Normal diet, Ca not ppted	No histamine detectable			
Normal diet, Ca ppted	12	0·65	0·13	<0·001
'Diet E' Ca ppted	9	5·23	0·68	0·15
'Diet E', Ca not ppted	10	8·01*	1·73	

* Value from Fig. 1.

Interference by calcium during histamine extraction. In order first to compare the usual (TCA) method of extraction with the newly devised method, using H_2SO_4 for preventing loss of histamine through precipitation of calcium dissolved by the TCA, the pooled bones from twelve normal and nine 'Diet E' rats were extracted by the new method and the results compared with the values from ten other rats fed on 'Diet E' and extracted with TCA alone. The subsequent assays indicate that while the mean histamine contents of the 'Diet E' bones do not differ significantly from one another, whether or not calcium has been first removed, they do differ significantly from the histamine values from normal bones (Table 1).

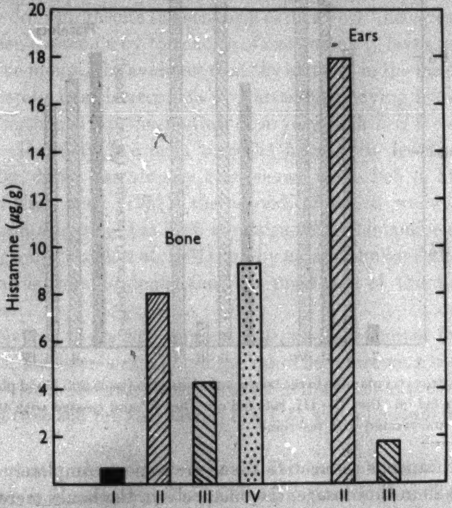
Histamine and 5-HT in 'Diet E' bones. In a preliminary experiment, using ten 'Diet E' and seven control rats, histamine and 5-HT were extracted from the pooled bones of each group, and histamine also from the pooled ears, and assayed. Although no statistical analysis of the results is possible, there is an obvious increase in the total histamine and 5-HT contents of the 'Diet E' bones, whereas the histamine values for ears remain virtually unchanged (Table 2). Since the mastocytosis in the treated animals occurs in the bones and not in the ears the results suggest that it is the mastocytosis which is responsible for the increase in histamine and 5-HT in the bones of the treated animals. In later experiments histamine was determined separately for each rat, permitting statistical evaluation of the results. The small weight of bone available per rat precluded individual assays for 5-HT.

TABLE 2. Histamine and 5-HT content of bones from normal and 'Diet E' rats. Preliminary experiment: bones from individual rats in each group pooled

Tissue	Diet	Histamine (μg/g)	5-HT (μg/g)
Ears	Normal	24·6	—
Ears	'Diet E'	20·1	—
Bones	Normal	1·44	0·009
Bones	'Diet E'	11·5	0·235

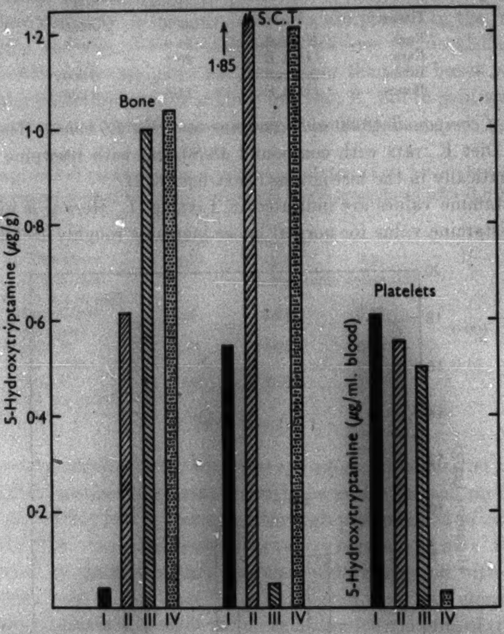
Effects of compound 48/80 *and reserpine on 'Diet E' bones.* The results of treating 'Diet E' rats with compound 48/80 and with reserpine are shown diagrammatically in the histograms (Text-figs. 1, 2).

The histamine values are indicated in Text-fig. 1. Here it is evident that the low histamine value for normal bones increases roughly 16-fold with the development of the syndrome caused by 'Diet E' and that this value is approximately halved by a subsequent 5-day course of compound 48/80. Statistical analysis shows a high degree of significance ($P<0{\cdot}001$) between the histamine values for normal and 'Diet E' bones and near significance ($P=0{\cdot}075$) between the 'Diet E' bones before and after compound 48/80. Thus the effect



Text-fig. 1. Histamine in rat bones and ears: I, normal rats; II, rats fed on 'Diet E'; III, rats fed on 'Diet E' and treated with 48/80; IV, rats fed on 'Diet E' and treated with reserpine.

of compound 48/80 on the bones is less than on the ears from the same rats, in which a precipitous fall occurs from a high initial value almost to the point of complete depletion. As previously stated, this initial high value for ears is characteristic of the rat and remains unaffected by 'Diet E'. Corresponding changes are seen in the mast cells. Whereas the mast cells in ears and subcutaneous connective tissue are almost completely destroyed by compound 48/80 in this dosage, the mast cells in the bones merely show some degranulation and stain less strongly with toluidine blue.



Text-fig. 2. 5-Hydroxytryptamine in rat bones, subcutaneous tissue and blood platelets: I, normal rats; II, rats fed on 'Diet E'; III, rats fed on 'Diet E' and treated with 48/80; IV, rats fed on 'Diet E' and treated with reserpine.

In contrast to the effects of compound 48/80, reserpine is without effect on the high histamine content of the 'Diet E' bones, the slight rise shown in Text-fig. 1 being devoid of statistical significance. There are no visible changes in the mast cells.

The 5-HT values are shown in Text-fig. 2, these being obtained for pooled tissues from the various groups. As with histamine, there is almost a 16-fold increase in the 5-HT content of the bones with the development of the 'Diet E' syndrome and this value tends to increase even further following treatment with either compound 48/80 or reserpine. Comparative values for subcutaneous connective tissue are interesting: here the 5-HT value also rises during dieting and is unaffected by reserpine, but it is greatly reduced by treatment with compound 48/80 which, as mentioned above, breaks up the mast cells of the skin and liberates their 5-HT. Values for the 5-HT in blood platelets were obtained for the four groups; as the histogram shows, there is little change in the 5-HT value of the platelets with dieting, nor is the value altered by compound 48/80. However, reserpine almost completely removes the 5-HT from the platelets, thus confirming its specific action.

DISCUSSION

The object of the investigation was to determine whether or not the tissue mast cells of the mouse and rat contain 5-HT as well as histamine. It is now clear that they do. Our results thus confirm earlier work and certain objections to this have been overcome by the choice of exceptionally favourable material.

As regards the mouse, the evidence that the increase in the mast cell content of the precancerous dermis accounts for the accompanying increase in 5-HT is greatly strengthened by the finding of a very high 5-HT content in the mastocytoma. Recently two such tumours have been described. The first (Dunn & Potter, 1957) has already been shown to be rich in histamine and 5-HT by Sjoerdsma *et al.* (1957): the second, of which we were fortunate enough to obtain a sample, has been investigated for histamine and 5-HT by Furth and his group (Furth *et al.* 1957) and by us, and similar results have been obtained. There is thus no doubt that the mast cells of the mouse are rich in both amines.

Until recently, no tissue rich in mast cells, and yet remote from both gut and epithelium, has been available in the rat. The interesting bone syndrome described by Urist & McLean (1957) thus offered a fresh approach for testing the hypothesis that the rat also carries 5-HT as well as histamine in its mast cells.

Curiously enough, these mast cells in the metaphysis of rachitic rats developing osteitis fibrosa do not respond so well as do the mast cells of the skin to the specific histamine-liberator, compound 48/80. In this they resemble the mast cells of the mesentery which liberate their histamine when 48/80 is injected intraperitoneally, but fail to do so when it is injected subcutaneously (P. B. Marshall, unpublished observation). This again poses the problem of how exactly the liberator acts in releasing histamine.

From the present standpoint, we can say that the small release of histamine and the lack of release of 5-HT from the bones by compound 48/80 were paralleled by the comparatively minor changes in the metaphyseal mast cells. In contrast, mast cells as well as histamine disappeared almost completely from the ears of the same rats. Reserpine, which strips the blood platelets of their 5-HT, failed to change the appearance of the mast cells in any situation and there was no significant change in their content of 5-HT.

It is thus clear, in accord with earlier suggestions, that the tissue mast cells of the mouse and rat are rich in both histamine and 5-HT, and that in containing 5-HT they differ from the mast cells of all other species which have so far been investigated.

SUMMARY

1. The contents of histamine and 5-hydroxytryptamine (5-HT) have been measured (*a*) in the dermis of mice treated with a carcinogenic hydrocarbon, (*b*) in a transplantable mouse mast cell tumour, and (*c*) in the bones of calcium-deficient rats.

2. In each case local increase in tissue mast cells has been found to be accompanied not only by an increase in histamine, but also by an increase in 5-HT.

3. The effects of the histamine-liberator, compound 48/80, and the 5-HT releaser, reserpine, were also tested on calcium-deficient rats. Compound 48/80 proved much less effective in removing histamine from the bones than from the skin, and the degree of disruption of the mast cells was comparable. Reserpine failed to alter either the 5-HT content or the mast cells in these bones.

4. The mast cells of the mouse and rat are exceptional in containing 5-HT as well as histamine and heparin.

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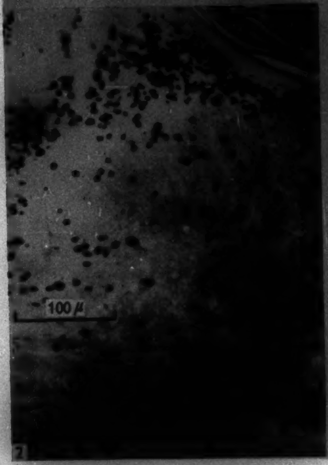

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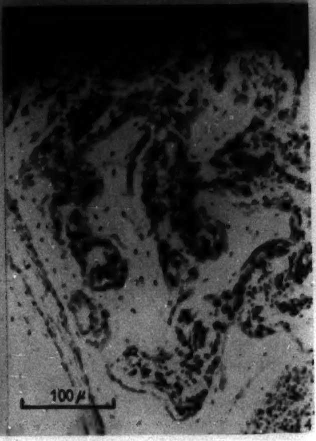

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EXPLANATION OF PLATE

Fig. 1. Section through normal mouse skin, stained toluidine blue. Scattered mast cells (dark spots) are seen mainly in the upper dermis.

Fig. 2. Section through precancerous mouse skin, stained as in Fig. 1. The surface epithelium, top right, is keratinized and the dermis is broadened. The outstanding feature is the great increase in the number of tissue mast cells.

Fig. 3. Vertical section through metaphysis of normal rat. Only the cartilage stains deeply with toluidine blue. There are no mast cells.

Fig. 4. Comparable section to Fig. 3, from a rachitic ('Diet E') rat. The epiphyseal plate, top, stains darkly as before. Note, however, the numerous mast cells which appear as dark spots between the altered bone trabeculae.

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THE ACTION OF TUBOCURARINE AND ACETYLCHOLINE ON CILIARY MOVEMENT

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A recent paper (Hill, 1957) described experiments in which tubocurarine and acetylcholine were tested on ciliary movement in the frog's oesophagus and in the rabbit trachea. The author found that tubocurarine had no effect even in concentrations as great as 0·2%, and that acetylcholine slightly increased ciliary activity only when concentrations not less than 0·02% were used. These results are at variance with the findings obtained on the same preparations by Kordik, Bülbring & Burn (1952), who observed that tubocurarine had an inhibitory action in concentrations as low as 10^{-6} g/ml., and that acetylcholine had a stimulant action in concentrations from 10^{-5} to 2×10^{-5} and an inhibitory action in concentrations higher than this. To throw light on the disagreement, further experiments have been carried out on the frog oesophagus, because this preparation is simpler to use and because most of Hill's work appears to have been done with it.

METHODS

The frog was placed inside a Perspex box, which has been described elsewhere (Burn, 1952). Before dissecting the frog two pieces of cotton wool soaked in water were placed in the box, together with a small thermometer; the temperature was kept at about 20° C throughout the experiment by replacing the pieces of cotton wool with pieces soaked in warmer or colder water as required.

The frog was decapitated but leaving the lower jaw intact. The spinal cord was destroyed and the frog was pinned dorsal surface uppermost on to a small cork mat. The skin was slit along the mid-dorsal line and the dorsal body wall was removed almost as far back as the sacral hump, taking care not to cut the dorsal oesophageal wall. The oesophagus was then cut down the mid-dorsal line and the cut continued a short distance into the stomach wall. The oesophagus was pinned out as a roughly rectangular sheet, as shown in Fig. 1. It was pulled out taut by pins at the four 'corners', and by other pins if necessary. It was usually raised a little above the underlying organs by placing small pieces of cotton wool soaked in frog Ringer's solution under the lower jaw and by keeping the oesophageal membrane well up and near the heads of the pins.

For determining the rate of ciliary movement poppy seeds of selected size were employed as previously described. A fine camel-hair brush was moistened with frog Ringer's solution and used to pick up the seeds one at a time and to place them on the mucous membrane. They were then carried by the cilia towards the stomach. Each seed was timed from the moment it moved under the slit in the lid of the box and down the oesophagus until it reached the finishing line marked on the lid. Care was taken not to stroke the oesophagus with the brush, as this stimulated ciliary movement. The mucous membrane was kept moist throughout the experiment with frog Ringer's solution applied gently from a pipette at regular intervals. The frog Ringer's and test solutions were kept at the same temperature as the inside of the box. If the stomach was not slit open for a short distance, mucus tended to accumulate at the lower end of the oesophagus during the course of the experiment and to slow down the ciliary movement.

When the preparation was ready, ten seeds were timed to make sure that the cilia were beating after the dissection. The rate was calculated from the mean time for ten seeds to travel the course. Out of each group of ten seeds, five were placed on one side of the mid line and five on the other, since there was a considerable variation across the width of the oesophagus. The solution to be tested was then applied.

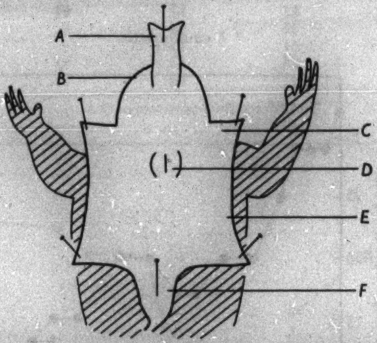

Fig. 1. Preparation of frog. See text. *A*, the tongue is pulled out and pinned; *B*, put moist cotton-wool under lower jaw here; *C*, keep oesophageal membrane taut and pulled up near heads of pins; *D*, glottis; *E*, pins may also be placed here and on opposite side; *F*, open the stomach to allow room to remove mucus.

RESULTS

Effect of tubocurarine 10^{-6} *g/ml.* In the first group of experiments the effect of tubocurarine 10^{-6} g/ml. was tested and in one experiment 10^{-5} g/ml. The results are shown in Table 1 and the details of one experiment are given in Fig. 2. The figures in Table 1 for the initial rate were the average of the first three consecutive mean rates obtained after setting up the preparation. After the tubocurarine was applied, observations were made for a period of 30–40 min. In all trials the rate of movement decreased, although the time for the slowest rate to be reached varied in different preparations. In none of these experiments was there complete recovery of the rate after washing off the tubocurarine even when the washing was continued for 50 min, and it was therefore doubtful whether the slowing of the rate was due to the tubocurarine.

Three control experiments from the same batch of frogs showed a similar rapid falling off in the rate after the first 15 min. The frogs used in these experiments had been kept in a cold room at 4° C for 2–3 months, and were in poor condition.

TABLE 1. Action of tubocurarine on frogs stored at 4° C for a long period. Observations in May

Concn. of tubocurarine (g/ml.)	Initial rate (mm/100 sec)	Slowest rate in presence of tubocurarine (mm/100 sec)	Reduction (%)	Recovery after tubocurarine removal
10^{-6}	21	11	48	I*
10^{-6}	27	12	56	I
10^{-5}	26	12·5	52	I
10^{-6}	26	19	27	I
10^{-6}	31	10	68	I
10^{-6}	30	17	43	I

* I = incomplete.

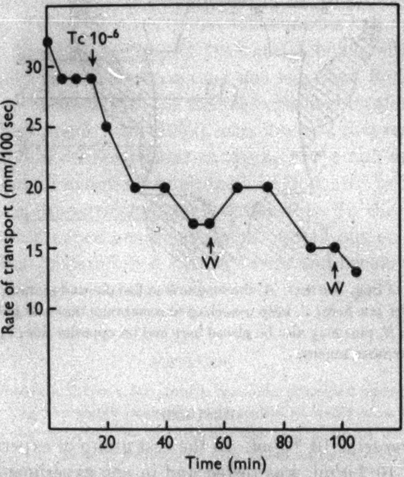

Fig. 2. Example of inconclusive observation made on the first frogs which were used. Ordinate, rate of transport of poppy seeds. At Tc, tubocurarine 10^{-6} g/ml. applied; at W it was removed by washing.

Effect of tubocurarine 10^{-4} *g/ml.* A fresh batch of frogs was obtained, and in control experiments on these it was found that the rate of poppy seed transport became steady 30–60 min after making the preparation. In all succeeding experiments the preparation was left for 30 min and then the rate was tested repeatedly during the next 30–40 min. If it remained steady, tubocurarine 10^{-4} g/ml. was applied. As shown in Table 2, this decreased the rate of transport, and after removing the tubocurarine the rate recovered to the initial value in a period which varied from 30 to 105 min (Fig. 3).

Testing of unknown solutions. The results given in Table 2 confirmed the earlier results obtained in this laboratory, but it was clear they would not be likely to convince others who could not repeat them elsewhere. In order to do this it was decided that a series of solutions should be tested some of which contained tubocurarine (10^{-4} g/ml.) and some which did not, the person testing (M.D.) not knowing which was which.

TABLE 2. Action of tubocurarine 10^{-4} g/ml. on fresh and active frogs. Observations in late May and June

Initial rate (mm/100 sec)	Slowest rate in presence of tubocurarine (mm/100 sec)	Reduction (%)	Time to slowest rate (min)	Recovery after tubocurarine removed	
					Time (min)
19	11	42	5	C*	immediately
22	12	45	20	I	—
17	12	30	15	C	105
16	9	44	25	C	70
21	9	57	15	C	30
29	12	58	40	C	65
22	13	40	10	C	60

* C = complete, I = incomplete.

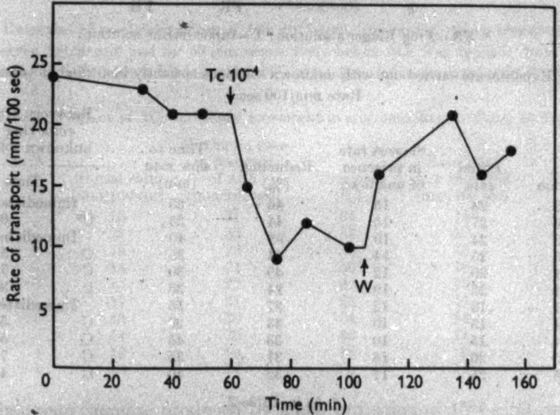

Fig. 3. Example of inhibitory effect of tubocurarine 10^{-4} g/ml. in fresh frogs. Note that the rate of ciliary transport was observed during 1 hr before tubocurarine was applied and that the rate recovered on washing it off. Conventions as for Fig. 2.

In the first series (series *A* in Table 3) only four out of six unknown solutions were correctly identified. One solution of tubocurarine was identified as frog Ringer's solution. It caused a decrease in the rate of transport, without return to the initial value when the solution was washed off; the decrease was therefore attributed to deterioration of the preparation. One solution which was frog Ringer's solution was identified as tubocurarine. Its effect was tested on three frogs. In one the result was inconclusive; in a second it produced a 54% reduction in rate, and in a third it caused a slight reduction in rate which did not alter after washing off the unknown.

TABLE 3. Testing of unknown solutions

Solution no.	No. of frogs used for test	Solution identified as	Solution was actually
A 1	1	FR	FR
2	2	T	T
3	2	T	T
4	3	T	FR
5	1	T	T
6	2	FR	T
B 1	3	T	T
2	4	FR	FR
3	2	FR	FR
4	3	T	T
5	2	T	T
6	2	FR	FR

* FR = Frog Ringer's solution; T = tubocurarine solution.

TABLE 4. Experiments carried out with unknown solutions correctly identified as tubocurarine. Rate mm/100 sec

Solution	Initial rate	Slowest rate in presence of unknown	Reduction (%)	Time to slow rate (min)	Recovery after removing unknown soln.	
						Time (min)
A 2	28	15	46	25	Immediate	
A 3	27	15	44	25	C*	60
A 3	24	10	58	40	Immediate	
A 5	25	14	44	35	C	55
B 1	20	12	40	30	C	75
B 1	25	19	24	35	C	80
B 1	19	12	37	55	Immediate	
B 4	15	10	33	20	C	55
B 4	15	10	33	45	C	50
B 5	26	18	31	35	C	70
B 5	25	17	32	40	C	45

* As in Table 2.

These results were not satisfactory, but experience was gained in the course of getting them, and a further series (series *B* in Table 3) of six unknown solutions was examined. Each was correctly identified. In Table 4 are given the details of the observations made with the six unknown solutions in the two series which were identified as, and actually were, tubocurarine.

The stimulant action of acetylcholine. With acetylcholine a stimulant action on ciliary movement was regularly seen when a concentration of 10^{-5} g/ml. was applied. This action was observed even with the first group of frogs which failed to respond clearly to tubocurarine. In Fig. 4 two examples are shown where the increase in the rate was about 50%, and where the rate dropped below the initial rate when the acetylcholine was removed. The effect is similar to that described previously by Kordik *et al.* (1952). The total number of experiments in which the stimulant action of acetylcholine was observed was ten. The mean increase produced by a concentration of 10^{-5} g/ml. was 50%, the smallest increase being 12% and the greatest being 79%. The figures for the maximum increase are shown in Table 5.

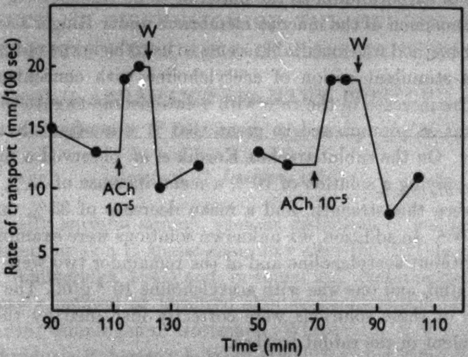

Fig. 4. Examples of excitatory effect of ACh 10^{-5} g/ml. The rate of ciliary transport had been observed for 90 min and for 50 min respectively before ACh was applied. Note that after washing off ACh the rate fell below the original, as observed in previous work. W, as in Fig. 2.

TABLE 5. Effect of ACh on ciliary movement in frog oesophagus. Conc. 10^{-5} g/ml.

Initial rate (mm/100 sec)	Maximum rate in presence of ACh (mm/100 sec)	Increase (%)	Rate after ACh removed (mm/100 sec)
16	31	94	11
21	32	53	19
15	23	53	16
14	21	50	16
14	27	92	16
17	21	24	17
13	20	54	14
14	20	43	11
12	19	58	9
15	24	60	14

DISCUSSION

The present observations on tubocurarine confirm the earlier observations from this laboratory (Kordik *et al.* 1952) except that in the earlier observations a concentration of 10^{-6} g/ml. was used, whereas in the present observations it was 10^{-4} g/ml. We think it is possible that this difference may be explained by the time of year, for the earlier observations were made in late September and October, while the present ones were made chiefly in June.

We cannot be sure of the reason for the failure of Dr Hill to observe the inhibitory effect of tubocurarine on ciliary movement. We believe it to be due to the choice of carborundum as the material to be transported, and perhaps also to the immersion of the mucous membrane under Ringer's solution. Our view that her method was unsuitable seems to us to be borne out by her failure to observe a stimulant action of acetylcholine with concentrations below 0·2 mg/ml. The increase in the rate with solutions one-twentieth of this concentration was so prompt and so great that it was often obvious without measurement. On the rabbit trachea Kordik *et al.* observed a mean increase of 27% on applying a solution of 10^{-5}, a mean increase of 33% on applying a solution twice this strength, and a mean decrease of 33% on applying a solution of 10^{-4}. In addition, six unknown solutions were examined, of which three were without acetylcholine and of the remainder two were with acetylcholine 10^{-5} g/ml. and one was with acetylcholine 10^{-4} g/ml. The composition and strength of these solutions were correctly identified by their effect on ciliary movement in the rabbit trachea.

SUMMARY

1. A further study has been made of the action of tubocurarine on the rate of transport of poppy seeds on the ciliated mucous membrane of the frog oesophagus. In observations on fresh and active frogs, tubocurarine in a strength 10^{-4} g/ml. decreased the rate of ciliary movement by 30–59% in seven consecutive experiments. In six of these the rate of ciliary movement returned to its initial value when the tubocurarine was removed.

2. The inhibition of ciliary movement by tubocurarine was then used to distinguish between unknown solutions which were either tubocurarine or frog saline. In a first series of six solutions four were correctly identified; in a second series of six solutions all were correctly identified. At the end of the investigation of tubocurarine, its inhibitory effect had been observed in 17 out of 18 experiments, with full recovery when the tubocurarine was removed.

3. The stimulant action of acetylcholine was observed in ten experiments using a concentration of 10^{-5} g/ml. The stimulation was often obvious on inspection.

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J. Physiol. (1958) 141, 527–534

THE EFFECT OF DISTENSION OF THE UTERUS ON THE COMPOSITION OF URINE OF CONSCIOUS DOGS

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Certain potentially harmful stimuli, such as distension of hollow viscera or pinching of viscera or skin in spinal man, may alter the rhythm of the heart (Guttmann & Whitteridge, 1947) and, in spinal and anaesthetized animal or man may cause a general or localized skin vasoconstriction (Downman & MacSwiney, 1946; Cunningham, Guttmann, Whitteridge & Wyndam, 1953). In normal individuals Robertson & Wolff (1950) found it difficult to demonstrate any appreciable rise in blood pressure with distension of the rectum, but they did not look for the skin vasoconstriction which almost certainly occurred (Carmichael, Doupe, Harper & MacSwiney, 1939), nor therefore for compensatory vasodilatation in other areas. Owing to the intrinsic difficulties of acquiring information, little is known regarding the existence of viscero-visceral reflexes comparable with the viscero-cutaneous reflexes. Mukherjee (1957*a*, *b*) showed in chloralosed cats whose buffer nerves were cut that renal vasoconstriction accompanied the rise in blood pressure which followed distension of the bladder, but that when the buffer nerves were intact the rise in blood pressure was small and the renal vasoconstriction more often absent than present. The renal vascular changes, then, are a part of the general cardiovascular response, and in the animal with intact nervous system the stabilizing mechanisms profoundly modify this vascular response. Mukherjee (1957*a*, *b*) did not determine whether the renal vasoconstriction he observed was general or restricted to a part of the kidney. Using the method of serial angiography, Trueta, Barclay, Daniel, Franklin & Prichard (1947) showed that in rabbits trauma and haemorrhage was followed by renal cortical vasoconstriction with a persisting blood flow in the renal medulla. They made no observations on the effect of distension of viscera. Daniel, Peabody & Prichard (1951, 1952) found that a renal cortical vasoconstriction could be induced in the cat, dog and sheep as well as in the rabbit. Franklin (1952) and Sophian (1955) have suggested that in pregnancy uterine tension may initiate utero-renal reflexes which bring about a renal cortical ischaemia, which then leads to the condition of pre-eclampsia. A serious criticism of the work of the last two authors is that they made no tests of renal function and that many of their conclusions are based on the appearance of the kidneys of animals under anaesthesia. As Daniel *et al.* (1952) point out, surface pallor of the kidney may be due to a general renal vasoconstriction as much as to vasoconstriction localized to the renal cortex. Further, neither oliguria nor albuminuria are pathognomic of renal cortical vasoconstriction. Since the problem is an important one, the renal function was tested in conscious dogs during periods when the uterus was put under tension by the sudden inflation of an intra-uterine balloon. A preliminary account of some of the results has already appeared (Pickford, 1956).

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METHODS

Dogs were prepared for observation by operations at which the external urethral orifice was exposed by a dorsal slit of the perineum, and at which the upper end of one uterine horn was divided from the ovary and fimbria of that side, brought through to the surface of the flank and stitched in place. No observations were made until healing was complete. The posterior lobe of the pituitary was removed transorally. The balloon for insertion into the uterus was joined by polythene tubing to a hand pump and pressure bottle and a recording Hg manometer, as described by Abrahams & Pickford (1954). The balloon and tubes were filled with 0·9% NaCl solution. With this system it was possible to raise the pressure in the balloon to the desired height in 3–4 sec or less. On the days on which they were to be used, the dogs were given a hydrating dose of water at noon and observations made after a second oral dose of water given at 2.15–2.30 p.m. of the same day. When the renal clearances of creatinine and diodone were to be measured, 3·5–4 g creatinine was given by mouth with the afternoon dose of water, and 6–9 ml. 30% diodone solution injected subcutaneously half an hour before the water was given. In general the methods used were similar to those described by Ali, Cross & Pickford (1958). The diet was maintained fairly, but not exactly, constant. At present no useful comments can be made on the higher post-operative electrolyte loss in the dog which was ovariectomized; in any case, this increase does not directly affect the question under investigation. Estimations of Na and K were made on a flame photometer, chloride by the method of Prout-Winter as described by Cole (1919), creatinine in the blood and urine by the methods of Rehberg (1926) and Folin (1914), respectively, and diodone by the method of Alpert (1941). After death the kidneys were examined histologically and all were found to be normal. Serial sections of the hypothalamus and pituitary confirmed that in the one dog the posterior lobe of the pituitary had been removed at operation.

RESULTS

Control observations showed that in the three dogs used the excretion rates of water, Na, K and Cl were normal; that is, the peak of diuresis was attained in 45–60 min, 80–90% of the water given was excreted in approximately 2 hr, and the excretion rates of Na, K and Cl fell steadily as diuresis progressed (Ali *et al.* 1958). Having established these facts, the effects of uterine distension were then investigated in the anoestrous dog, the oestrogen-treated dog and a dog whose posterior pituitary lobe had been removed; examination was also made of the effect of distension of the lower segment of the uterus.

Effect of uterine distension in the anoestrous dog. It was first determined that distension of the uterus during, and for a time after, the administration of water, in no way altered the normal course of diuresis. Ten experiments were then performed, six experiments being on one dog (Anona, 9·5 kg) and four on another (Maggs, 20 kg). The effect of uterine distension was examined under two sets of conditions. In the first the balloon was inserted and inflated at the peak of diuresis; and in the second, the balloon was inserted immediately following the administration of water, but again inflated only at the peak of diuresis. The pressure to which the balloon was initially inflated was from 30 to 100 mm Hg. Left to itself the intra-uterine pressure declined steadily, but the effects on renal function were no different when this fall was prevented by the repeated addition of small volumes of saline to the uterine bag. The results were also unaffected by the time of insertion of the uterine balloon. In seven of the ten experiments inflation of the bag produced no alteration in the course of the water diuresis, nor any deviation from normal in the excretion of Na or K (Fig. 1*A*, Anona). In one experiment uterine distension caused a brief increase in the rate of urine excretion with perhaps a slight delay in the usual fall of Na excretion. In two other experiments on the same dog the urinary excretion rate fell somewhat and the diuresis continued at the lower level (Fig. 1*B*). On one of these last two occasions renal clearances were measured and it was found that although the initial fall in

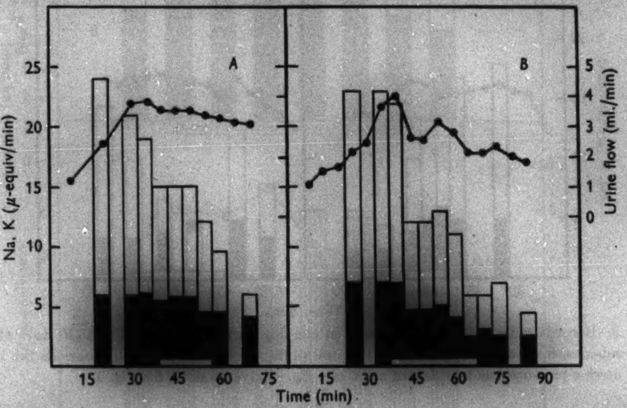

Fig. 1. Records of two observations made on one dog. In both *A* and *B* the continuous line shows the rate of urine flow in ml./min. The histograms show the excretion rate of Na (open columns) and K (black columns) in μ-equiv/min. The open blocks along the abscissa show the time during which the uterus was distended. In *A* the pressure applied was 40 mm Hg, and in *B*, 30 mm Hg. On both occasions 250 ml. water was given by mouth at zero time.

urinary excretion rate was accompanied by a fall in both glomerular filtration rate (24%) and renal peak flow (42%), within 15 min urine flow and clearance values had returned to normal levels.

Effect of uterine distension after treatment with stilboestrol. It was considered possible that the anoestrous uterus had a low sensitivity to a rise in tension. Experiments were therefore performed on Anona and Maggs after they had been injected with stilboestrol diproprionate (0·35 and 0·75 mg, respectively) in oil. Inspection at laparotomy had previously shown that within 24 hr of the subcutaneous injection of stilboestrol the dog uterus becomes enlarged and highly vascular. Observations were accordingly made on the two days following injection. In Anona uterine distension 24 hr after stilboestrol administration caused a small rise in the rate of urine flow, but had no significant effect on the normal course of Na and K excretion (Fig. 2*A*). Two days after the injection a transient decrease in the rate of urine flow followed inflation of the uterine bag, and there was some fall in the rate of Na loss (Fig. 2*B*). In Maggs uterine distension 24 hr after the injection of stilboestrol caused a sharp reduction in water, Na and K loss, but excretion returned to the original level in 7 min. The following day uterine distension was without effect on renal excretion.

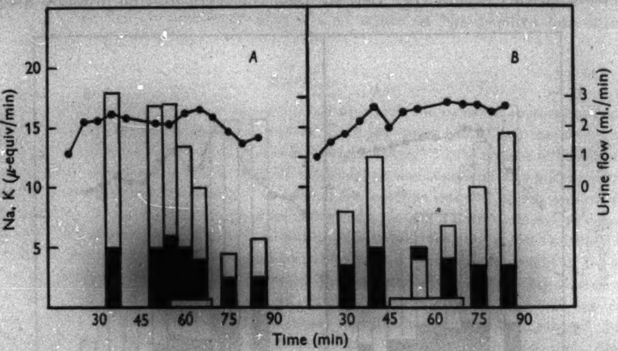

Fig. 2. Records of two observations made on one dog on the 1st (*A*) and 2nd (*B*) days after a subcutaneous injection of 0·35 mg stilboestrol diproprionate. On both occasions the intrauterine pressure was maintained at 50 mm Hg. Conventions as for Fig. 1.

Effect of distension of the lower segment of the uterus. It is known that the different parts of the uterus vary in sensitivity (see Fitzpatrick, 1957). In the third dog, Little Claus, which had been ovariectomized 12 days previously and whose uterus was showing spontaneous contractions, care was taken to push the balloon as far down towards the cervix as possible. Post-mortem measurements showed that the tip of the bag must have been close to the cervical orifice. When diuresis was established, a pressure of 140–150 mm Hg was thrown into the balloon and maintained for 1 hr. This high intra-uterine pressure did not alter the normal pattern of water excretion nor the already low rate of Na and K loss, but after some delay the rate of Cl excretion rose for a time (Fig. 3).

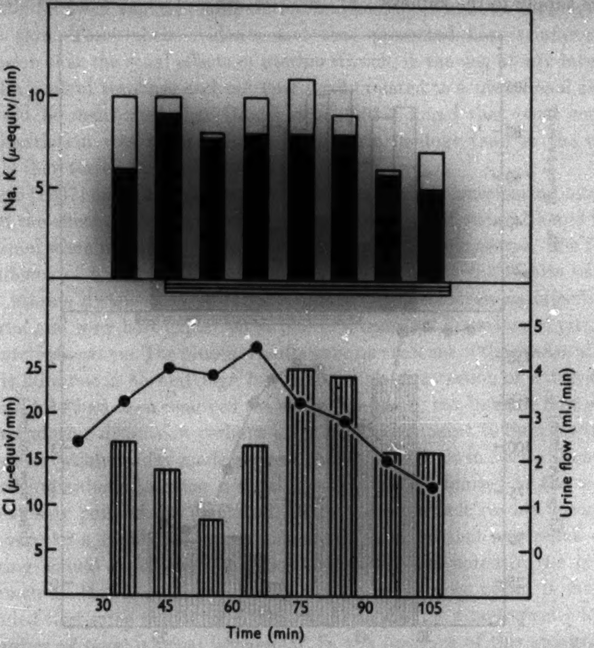

Fig. 3. Effect of distending the lowest part of one uterine horn with a pressure of 150 mm Hg during the period shown by the horizontally lined block. Lower part of graph shows rate of urine flow (continuous line) and Cl loss (columns with perpendicular lines). Upper part of graph shows rate of Na (open columns) and K loss (black columns). 300 ml. water given by mouth at zero time.

Effect of uterine distension after removal of the posterior lobe of the pituitary. Since Dale's observations in 1909 it has been known that the posterior lobe of the pituitary contains a substance which profoundly affects uterine activity. There is no suggestion that this substance directly affects uterine sensitivity. The posterior lobe also contains the vasopressor-antidiuretic factor. Since the opportunity offered, it seemed worth while re-examining Little Claus whose posterior lobe had been removed 11 days previously. The water intake had returned to normal levels. The uterus still showed spontaneous activity; the renal nerves were intact. In Fig. 4 it can be seen that after raising the pressure in the uterine bag to 150 mm Hg the rate of urine flow increased for a while, and the excretion rates of Na and K rose also, but Cl loss fell. None of these changes persisted despite the maintenance of uterine distension and the intact nerve supply to the kidneys.

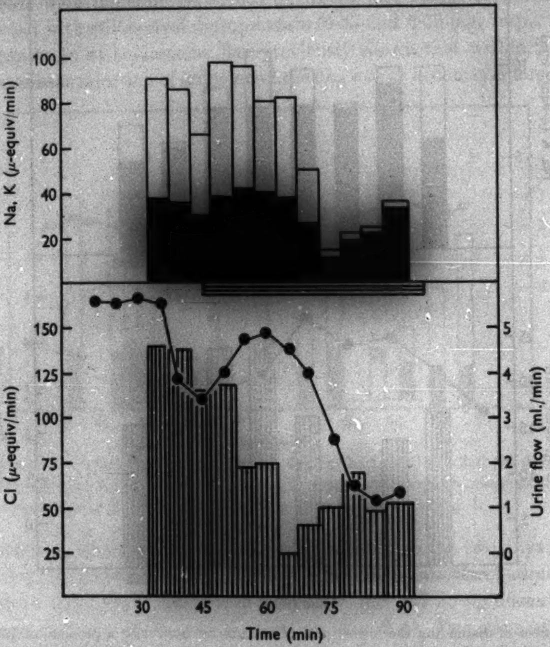

Fig. 4. Observation on same dog as in Fig. 3, but 11 days after removal of posterior lobe of pituitary. Intra-uterine pressure, 150 mm Hg. Conventions as for Fig. 3

DISCUSSION

The results described above suggest that in the dog, whether normal and anoestrous, treated with stilboestrol or ovariectomized, a utero-renal reflex is not highly active, even when a pressure as great as 150 mm Hg is applied in the uterus. The results provide no evidence that a rise in intra-uterine tension causes vasoconstriction localized to the renal cortex, and the inconstant alterations seen in renal function could well be due to variable vascular changes occurring in the whole kidney, these being a part of the general vascular response to distension of a hollow viscus as described by Mukherjee (1957 *a*, *b*).

Two sets of workers have previously made observations on changes in renal function when the uterus is under tension. McGaughey, Weller & Anslow (1956), using dogs under chloralose anaesthesia, noted that uterine insufflation caused a 23% reduction in *p*-aminohippuric acid clearance; but a similar reduction followed vaginal manipulation and non-specific sensory stimulation of the skin. Thus, their evidence and that presented here reinforce the impression that the renal effects of uterine stretch, in the dog at any rate, are part of a general response and not specifically related to a utero-renal reflex. It should be mentioned that Daniel *et al.* (1952) found that renal cortical vasoconstriction was more difficult to produce in dogs than in the other animals they tested.

Saurio (1957) made a large number of observations on conscious rabbits and studied the effect of uterine distension on the excretion of water, Na and K by the animal when normal and after section of the splanchnic nerves. She found no evidence of a utero-renal reflex, and concluded that the results seen to follow uterine distension could be matched by the intravenous injection of pituitrin, and were best explained as due to a transient liberation of pituitary antidiuretic hormone. The present results gave no evidence of the release of antidiuretic hormone in normal dogs, but in the single observation on a dog whose posterior lobe had been removed the sharp temporary rise in urine flow which followed uterine distension perhaps gives negative support to Saurio's thesis.

All observations so far made suggest that it is difficult in normal unanaesthetized individuals to cause a renal vasoconstriction limited to the cortex. As previously pointed out (Pickford, 1956) uterine muscle readily accommodates even to a rapid rise in intra-uterine tension, so that any reflex effect occurring should be of short duration, as indeed was noted in the present experiments. However, since renal cortical necrosis does occur, it must be concluded that some unidentified abnormal condition is a prerequisite for the appearance of renal cortical ischaemia. In the presence of this abnormality uterine stretch may be one of a number of adequate stimuli.

SUMMARY

1. Observations were made on conscious dogs in which one uterine horn was fistulated. Uterine movements and the urinary loss of water, Na and K were noted before and after the sudden inflation of an intra-uterine balloon.

2. The dogs were tested when normal, after the administration of stilboestrol, after ovariectomy, and after ovariectomy accompanied by removal of the posterior lobe of the pituitary.

3. No consistent effect followed inflation of the intra-uterine balloon, and ten out of sixteen times renal activity was unchanged.

4. The results provided no evidence that, in dogs, a utero-renal reflex causes renal cortical vasoconstriction.

Addendum. Since this paper was written four observations have been made on the response to uterine distension (pressure, 40 mm Hg) of a dog with diabetes insipidus (induced by transoral section of the supraopticohypophysial tracts). Water and K excretion showed no discernible pattern of response; on every occasion Na excretion fell when the uterine bag was inflated; on all occasions Cl excretion remained steady.

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CONTENTS OF VOLUME 64.

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CONTENTS OF VOLUME 141

No. 1. 3 *April* 1958

PAGE

Reflex stimulation and inhibition of reticulum contractions in the ruminant stomach. By D. A. TITCHEN 1

Factors affecting histamine-stimulated gastric secretion in anaesthetized dogs. By J. W. BLACK, E. W. FISHER and A. N. SMITH 22

The effects of 5-hydroxytryptamine on gastric secretion in anaesthetized dogs. By J. W. BLACK, E. W. FISHER and A. N. SMITH 27

Reversible inactivation of nervous activity in a fish gut. By G. BURNSTOCK 35

Studies on the responses of the isolated nictitating membrane of the cat. By J. W. THOMPSON 46

Intestinal transfer of short-chain fatty acids *in vitro*. By D. H. SMYTH and C. B. TAYLOR 73

The response of the new-born puppy to water, salt and food. By R. A. MCCANCE and ELSIE M. WIDDOWSON 81

The response of the new-born piglet to an excess of potassium. By R. A. MCCANCE and ELSIE M. WIDDOWSON 88

The distribution of cholinesterase in the adrenal glands of the rat, cat and rabbit. By R. E. COUPLAND and R. L. HOLMES 97

The destruction of the antidiuretic activity of vasopressin by —SH active compounds. By S. E. DICKER and A. L. GREENBAUM . . . 107

The depression of the receptor potential in Pacinian corpuscles. By J. DIAMOND, J. A. B. GRAY and D. R. INMAN 117

The action of reserpine on the peripheral sympathetic system. By ERICH MUSCHOLL and MARTHE VOGT 132

The heat exchanges of wet skin. By D. McK. KERSLAKE and J. L. WADDELL 156

The relation between the coefficients for heat exchange by convection and by evaporation in man. By D. F. BREBNER, D. McK. KERSLAKE and J. L. WADDELL 164

Effects of acetylcholine on the activity of the lumbosacral cord of the cat. By A. FERNANDEZ DE MOLINA, J. A. B. GRAY and J. F. PALEER . 169

Electrolyte excretion in diuretic and non-diuretic dogs. By M. N. ALI, R. B. CROSS and MARY PICKFORD 177

The distribution of the catechol amines in homogenates of the bovine adrenal medulla. By N. R. EADE 183

No. 2. 30 *April* 1958

PAGE

Observations on the nature of the chromaffin reaction. By A. J. HALE 193

A study of rapid mechanical events in a mechano-receptor. By S. J. HUBBARD 198

The action of inhibitors on the facilitated hexose transfer system in erythrocytes. By FREDA BOWYER and W. F. WIDDAS 219

Muscular exercise on Mount Everest. By L. G. C. E. PUGH . . . 233

The influence of age on peripheral vasodilatation in a hot environment. By R. F. HELLON and A. R. LIND 262

The interaction of pH, tonicity and electrolyte concentration on the motility of dog spermatozoa. By R. G. WALES and I. G. WHITE . . 273

The effects of hexamethonium bromide on temperature regulation in man. By A. C. L. HSIEH 281

Some effects produced by adrenaline upon neuromuscular propagation in rats. By K. KRNJEVIĆ and R. MILEDI 291

Some properties of frog skin mechanoreceptors. By W. T. CATTON . 305

Renal function in the foetal and new-born guinea-pig. By J. W. BOYLAN, ELIZABETH P. COLBOURN and R. A. McCANCE 323

Antidiuresis in adult and old rats. By S. E. DICKER and JOAN NUNN. 332

Temporal and spatial summation in human vision at different background intensities. By H. B. BARLOW 337

Anion interaction in frog muscle. By E. J. HARRIS. 351

The effect of changes in ion concentration on ventricular fibrillation induced electrically. By N. T. KÄRKI 366

No. 3. 28 *May* 1958

The relation of potassium deficiency to muscular paralysis by insulin. By F. G. J. OFFERIJNS, D. WESTERINK and A. F. WILLEBRANDS . . 377

Contraction in molluscan smooth muscle. By B. C. ABBOTT and J. LOWY 385

Mechanical properties of *Helix* and *Mytilus* muscle. By B. C. ABBOTT and J. LOWY 398

'Breath-holding' measurement of carbon monoxide diffusing capacity. Comparison of a field test with steady-state and other methods of measurement. By R. J. SHEPHARD 408

PAGE

Factors influencing limb temperature during experiments on skeletal muscle. By Brenda Bigland and Eleanor Zaimis 420

The effect of lowered muscle temperature on the action of neuromuscular blocking drugs. By Brenda Bigland, Beryl Goetzee, Jennifer Maclagan and Eleanor Zaimis 425

The excitation of Renshaw cells by pharmacological agents applied electrophoretically. By D. R. Curtis and Rosamond M. Eccles . . 435

The effect of diffusional barriers upon the pharmacology of cells within the central nervous system. By D. R. Curtis and Rosamond M. Eccles 446

Membrane potentials recorded with high-resistance micro-electrodes; and the effects of changes in ionic environment on the electrical and mechanical activity of the smooth muscle of the taenia coli of the guinea-pig. By Mollie E. Holman 464

Spinal tracts and supraspinal centres influencing viscero-motor and allied reflexes in cats. By C. B. B. Downman and A. Hussain . . 489

Cardiovascular responses to electrical stimulation of the brain stem. By Clarence N. Peiss 500

5-Hydroxytryptamine and histamine in mast cells of the mouse and rat. By Rosemary Cass, P. B. Marshall and J. F. Riley 510

The action of tubocurarine and acetylcholine on ciliary movement. By J. H. Burn and Margaret Day 520

The effect of distension of the uterus on the composition of urine of conscious dogs. By V. C. Abrahams and Mary Pickford 527

PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

11 *January* 1958

PAGE

Creese, R. and Hashish, S. E. E. Collection of labelled substances from muscle . 1*P*

Joyce, C. R. B. Measurement of reaction times with a conventional scaling unit . 1*P*

Cutting, Jane A. and D'Silva, J. L. A perfused rat hind-limb preparation . . 2*P*

Glaser, E. M. The retention of adaptation to warming and cooling of one hand . 3*P*

Bedford, T. H. B. The effect of tilting from the horizontal to the tail upwards position on the pressure of the cerebrospinal fluid of the dog 3*P*

Munro, A. F. and Robinson, R. Normal levels for plasma adrenaline and noradrenaline compared with those in subjects with complete transverse lesions of the spinal cord 4*P*

Creese, R., Hashish, S. E. E. and Scholes, N. W. Potassium in stimulated muscle . 5*P*

O'Connor, W. J. The renal response of conscious dogs to the ingestion of 0·9 % sodium chloride 6*P*

Thomas, J. G. An electrical method for recording eye movements . . . 7*P*

Nonweiler, T. The work production of man; studies on racing cyclists . . . 8*P*

Joyce, C. R. B. Differences between parts of packed human red cell column . 9*P*

Chalmers, T. M., Kekwick, A. and Pawan, G. L. S. Observations on the fat-mobilizing activity of human urine during periods of food deprivation . . 9*P*

Rodnight, R. Carbon monoxide saturation and the recovery of 5-hydroxytryptamine from whole blood 10*P*

Ullmann, Elisabeth. Osmotic diuresis during water diuresis 11*P*

Holmes, R. L. and Wolstencroft, J. H. Costocervical and thyrocervical contributions to the blood supply of the cat brain 12*P*

Holton, Pamela. The liberation of ATP from perfused rabbits' ears on antidromic stimulation of the sensory nerve 13*P*

Brittain, R. T. and Collier, H. O. J. Effects of 5-hydroxytryptamine and 5-hydroxytryptophane on defaecation and propulsion of a charcoal meal in intact mice 14*P*

21–22 *February* 1958

Bergel, D. H., McDonald, D. A. and Taylor, M. G. A method for measuring arterial impedance using a differential manometer 17*P*

Archer, H. E., Scowen, E. F. and Watts, R. W. E. Studies on primary hyperoxaluria 18*P*

Mendel, David. A method for the continuous determination of the pH of the blood 19*P*

Aumonier, F. J., Crosfill, M. L. and Widdicombe, J. G. The relationship between lung compliance and histology in different species 20*P*

Widdicombe, J. G. A method for recording changes in bronchial tone . . 20*P*

Bergel, D. H. A photo-electric method for the determination of the elasto-viscous behaviour of the arterial wall 22*P*

Taylor, M. G. A simple electrical computer for Fourier analysis and synthesis . 23*P*

Buller, A. J. and Styles, P. A simple method of time coding for use with continuous film records 25*P*

Hill, I. M., Hirt, R. L., Tubbs, O. S. and Wells, B. G. The use of a Gibbon type 'heart-lung' machine in experimental cardiac surgery 27*P*

PAGE

Lindop, Patricia J. and Rotblat, J. A method of recording whole-body movement of mice in relation to ageing 27*P*

Lindop, Patricia J. and Rotblat, J. Whole-body irradiation of animals by 15 MeV X-rays from the linear accelerator 27*P*

Donath, E. M., Hadfield, J. and Scowen, E. F. The response of the male mouse breast to human mastotrophic substances 27*P*

Cotes, J. E., Meade, F. and Wise, M. E. Variations, in the vertical work done against gravity and in the energy expenditure of walking, between men and women and in relation to training 28*P*

Hunt, J. N. and Murray, F. A. Changes in gastric function during pregnancy and lactation in women 29*P*

Benforado, J. M. Effect of thyroid state on the contractility of isolated rat ventricle strips 29*P*

Theobald, G. W. The separate release of oxytocin and ADH 30*P*

Hope, D. B. Cystathionine in brain and liver from pyridoxine-deficient rats . 31*P*

Daly, M. de Burgh and Scott, Mary J. The effects of stimulation of the carotid body chemoreceptors on heart rate in the dog 32*P*

Glaister, D. H., Hearnshaw, J. R., Heffron, P. F. and Peck, A. W. Gustatory sweating following parotidectomy 33*P*

Whitteridge, D. The motor supply to the extra-ocular muscle spindles . . . 33*P*

Harkness, Margaret L. R. and Harkness, R. D. Changes in the distensibility of the uterine cervix of the rat after parturition 34*P*

O'Connor, W. J. The renal response of conscious dogs to occlusion of the common carotid arteries 35*P*

Fletcher, J. G. Adaptation in man to exhausting physical work 35*P*

Wolff, H. S. The accuracy of the integrating motor pneumotachograph (IMP) . 36*P*

Lloyd, Sybil M. The responses of the vascular system in different phases of the reproductive cycle 37*P*

Andrews, W. H. H. and Butterworth, K. R. The vascular action of 5-HT on the canine liver 38*P*

Born, G. V. R. and Gillson, R. E. The effect of 2:4-dinitrophenol and of potassium on the uptake of 5-HT by platelets 39*P*

Lindop, Patricia J. and Rotblat, H. The variation of radiation sensitivity with age 39*P*

Cobbold, A. F., Galbraith, H. J. B., Ginsburg, Jean and Paton, A. Circulatory, metabolic and respiratory effects of *iso*propylnoradrenaline in man . . 40*P*

Andrew, B. L. and Barr, Margaret N. Anaesthesia and local tetanus intoxication 40*P*

Dimsdale, J. and Lockett, Mary F. A preliminary investigation of the effect of isoprenaline on the breathing of chloralosed cats 42*P*

Black, J. W., Fisher, E. W. and Smith, A. N. Factors affecting histamine-stimulated gastric secretion in anaesthetized dogs 42*P*

Currie, J. C. M. and Ullmann, Elisabeth. Polyuria during experimental modifications of breathing 43*P*

Aumonier, F. J., Cowan, G. A., Franklin, K. J. and Marsden, J. L. Variation in the response of human tissues to similar stimuli at different ages . . 45*P*

LIST OF AUTHORS

Papers published in Proceedings by title only are indicated by (T)

PAGE

Abbott, B. C. and Lowy, J. Contraction in molluscan smooth muscle . . 385

Abbott, B. C. and Lowy, J. Mechanical properties of *Helix* and *Mytilus* muscle. 398

Abrahams, V. C. and Pickford, Mary. The effect of distension of the uterus on the composition of urine of conscious dogs 527

Ali, M. N., Cross, R. B. and Pickford, Mary. Electrolyte excretion in diuretic and non-diuretic dogs 177

Andrew, B. L. and Barr, Margaret N. Anaesthesia and local tetanus intoxication 40*P*

Andrews, W. H. H. and Butterworth, K. R. The vascular action of 5-HT on the canine liver 38*P*

Archer, H. E., Scowen, E. F. and Watts, R. W. E. Studies on primary hyperoxaluria 18*P*

Aumonier. F. J., Cowan, G. A., Franklin, K. J. and Marsden, J. L. Variation in the response of human tissues to similar stimuli at different ages 45*P*

Aumonier, F. J., Crosfill, M. L. and Widdicombe, J. G. The relationship between lung compliance and histology in different species 20*P*

Barlow, H. B. Temporal and spatial summation in human vision at different background intensities 337

Barr, Margaret N. and Andrew, B. L. Anaesthesia and local tetanus intoxication 40*P*

Bedford, T. H. B. The effect of tilting from the horizontal to the tail upwards position on the pressure of the cerebrospinal fluid of the dog 3*P*

Benforado, J. M. Effect of thyroid state on the contractility of isolated rat ventricle strips 29*P*

Bergel, D. H. A photo-electric method for the determination of the elasto-viscous behaviour of the arterial wall 22*P*

Bergel, D. H., McDonald, D. A. and Taylor, M. G. A method for measuring arterial impedance using a differential manometer 17*P*

Bigland, Brenda and Zaimis, Eleanor. Factors influencing limb temperature during experiments on skeletal muscle 420

Bigland, Brenda, Goetzee, Beryl, Maclagan, Jennifer and Zaimis, Eleanor. The effect of lowered muscle temperature on the action of neuromuscular blocking drugs 425

Black, J. W., Fisher, E. W. and Smith, A. N. Factors affecting histamine-stimulated gastric secretion in anaesthetized dogs 22

Black, J. W., Fisher, E. W. and Smith, A. N. The effects of 5-hydroxytryptamine on gastric secretion in anaesthetized dogs 27

Black, J. W., Fisher, E. W. and Smith, A. N. Factors affecting histamine-stimulated gastric secretion in anaesthetized dogs 42*P*

Born, G. V. R. and Gillson, R. E. The effect of 2:4-dinitrophenol and of potassium on the uptake of 5-HT by platelets 39*P*

Bowyer, Freda and Widdas, W. F. The action of inhibitors on the facilitated hexose transfer system in erythrocytes 219

Boylan, J. W., Colbourn, Elizabeth, P. and McCance, R. A. Renal function in the foetal and new-born guinea-pig 323

Brebner, D. F., Kerslake, D. McK. and Waddell, J. L. The relation between the cofficients for heat exchange by convection and by evaporation in man . 164

PAGE

Brittain, R. T. and Collier, H. O. J. Effects of 5-hydroxytryptamine and 5-hydroxytryptophane on defaecation and propulsion of a charcoal meal in intact mice 14*P*

Buller, A. J. and Styles, P. A simple method of time coding for use with continuous film records 25*P*

Burn, J. H. and Day, Margaret. The action of tubocurarine and acetylcholine on ciliary movement 520

Burnstock, G. Reversible inactivation of nervous activity in a fish gut . . 35

Butterworth, K. R. and Andrews, W. H. H. The vascular action of 5-HT on the canine liver 38*P*

Cass, Rosemary, Marshall, P. B. and Riley, J. F. 5-Hydroxytryptamine and histamine in mast cells of the mouse and rat 510

Catton, W. T. Some properties of frog skin mechanoreceptors 305

Chalmers, T. M., Kekwick, A. and Pawan, G. L. S. Observations on the fat-mobilizing activity of human urine during periods of food deprivation . . 9*P*

Cobbold, A. F., Galbraith, H. J. B., Ginsburg, Jean and Paton, A. Circulatory, metabolic and respiratory effects of *iso*propylnoradrenaline in man . . 40*P*

Colbourn, Elizabeth P., Boylan, J. W. and McCance, R. A. Renal function in the foetal and new-born guinea-pig 323

Collier, H. O. J. and Brittain, R. T. Effects of 5-hydroxytryptamine and 5-hydroxytryptophane on defaecation and propulsion of a charcoal meal in intact mice 14*P*

Cotes, J. E., Meade, F. and Wise, M. E. Variations, in the vertical work done against gravity and in the energy expenditure of walking, between men and women and in relation to training 28*P*

Coupland, R. E. and Holmes, R. L. The distribution of cholinesterase in the adrenal glands of the rat, cat and rabbit 97

Cowan, G. A., Aumonier, F. J., Franklin, K. J. and Marsden, J. L. Variation in the response of human tissues to similar stimuli at different ages . . . 45*P*

Creese, R. and Hashish, S. E. E. Collection of labelled substances from muscle (T) 1*P*

Creese, R., Hashish, S. E. E. and Scholes, N. W. Potassium in stimulated muscle 5*P*

Crosfill, M. L., Aumonier, F. J. and Widdicombe, J. G. The relationship between lung compliance and histology in different species 20*P*

Cross, R. B., Ali, M. N. and Pickford, Mary. Electrolyte excretion in diuretic and non-diuretic dogs 177

Currie, J. C. M. and Ullmann, Elisabeth. Polyuria during experimental modifications of breathing 43*P*

Curtis, D. R. and Eccles, Rosamond M. The excitation of Renshaw cells by pharmacological agents applied electrophoretically 435

Curtis, D. R. and Eccles, Rosamond M. The effect of diffusional barriers upon the pharmacology of cells within the central nervous system 446

Cutting, Jane A. and D'Silva, J. L. A perfused rat hind-limb preparation . 2*P*

Daly, M. de Burgh and Scott, Mary J. The effects of stimulation of the carotid body chemoreceptors on heart rate in the dog 32*P*

Day, Margaret and Burn, J. H. The action of tubocurarine and acetylcholine on ciliary movement. 520

Diamond, J., Gray, J. A. B. and Inman, D. R. The depression of the receptor potential in Pacinian corpuscles 117

Dicker, S. E. and Greenbaum, A. L. The destruction of the antidiuretic activity of vasopressin by —SH active compounds 107

Dicker, S. E. and Nunn, Joan. Antidiuresis in adult and old rats . . . 332

PAGE

Dimsdale, J. and Lockett, Mary F. A preliminary investigation of the effect of isoprenaline on the breathing of chloralosed cats 42*P*

Donath, E. M., Hadfield, J. and Scowen, E. F. The response of the male mouse breast to human mastotrophic substances 27*P*

Downman, C. B. B. and Hussain, A. Spinal tracts and supraspinal centres influencing visceromotor and allied reflexes in cats 489

D'Silva, J. L. and Cutting, Jane A. A perfused rat hind-limb preparation . 2*P*

Eade, N. R. The distribution of the catechol amines in homogenates of the bovine adrenal medulla 183

Eccles, Rosamond M. and Curtis, D. R. The excitation of Renshaw cells by pharmacological agents applied electrophoretically 435

Eccles, Rosamond M. and Curtis, D. R. The effect of diffusional barriers upon the pharmacology of cells within the central nervous system 446

Fernandez de Molina, A., Gray, J. A. B. and Palmer, J. F. Effects of acetylcholine on the activity of the lumbosacral cord of the cat 169

Fisher, E. W., Black, J. W. and Smith, A. N. Factors affecting histamine-stimulated gastric secretion in anaesthetized dogs 22

Fisher, E. W., Black, J. W. and Smith, A. N. The effects of 5-hydroxytryptamine on gastric secretion in anaesthetized dogs 27

Fisher, E. W., Black, J. W. and Smith, A. N. Factors affecting histamine-stimulated gastric secretion in anaesthetized dogs 42*P*

Fletcher, J. G. Adaptation in man to exhausting physical work 35*P*

Franklin, K. J., Aumonier, F. J., Cowan, G. A. and Marsden, J. L. Variation in the response of human tissues to similar stimuli at different ages . . . 45*P*

Galbraith, H. J. B., Cobbold, A. F., Ginsburg, Jean and Paton, A. Circulatory, metabolic and respiratory effects of *iso*propylnoradrenaline in man . . . 40*P*

Gillson, R. E. and Born, G. V. R. The effect of 2:4-dinitrophenol and of potassium on the uptake of 5-HT by platelets 39*P*

Ginsburg, Jean, Cobbold, A. F., Galbraith, H. J. B. and Paton, A. Circulatory, metabolic and respiratory effects of *iso*propylnoradrenaline in man . . . 40*P*

Glaister, D. H., Hearnshaw, J. R., Heffron, P. F. and Peck, A. W. Gustatory sweating following parotidectomy (T) 33*P*

Glaser, E. M. The retention of adaptation to warming and cooling of one hand (T) 3*P*

Goetzee, Beryl, Bigland, Brenda, Maclagan, Jennifer and Zaimis, Eleanor. The effect of lowered muscle temperature on the action of neuromuscular blocking drugs 425

Gray, J. A. B., Diamond, J. and Inman, D. R. The depression of the receptor potential in Pacinian corpuscles. 117

Gray, J. A. B., Palmer, J. F. and Fernandez de Molina, A. Effects of acetylcholine on the activity of the lumbosacral cord of the cat 169

Greenbaum, A. L. and Dicker, S. E. The destruction of the antidiurectic activity of vasopressin by —SH active compounds 107

Hadfield, J., Donath, E. M. and Scowen, E. F. The response of the male mouse breast to human mastotrophic substances 27*P*

Hale, A. J. Observations on the nature of the chromaffin reaction 193

Harkness, Margaret L. R. and Harkness, R. D. Changes in the distensibility of the uterine cervix of the rat after parturition 34*P*

Harkness, R. D. and Harkness, Margaret, L. R. Changes in the distensibility of the uterine cervix of the rat after parturition 34*P*

Harris, E. J. Anion interaction in frog muscle 351

Hashish, S. E. E. and Creese, R. Collection of labelled substances from muscle (T) 1*P*

PAGE

Hashish, S. E. E., Creese, R. and Scholes, N. W. Potassium in stimulated muscle 5*P*

Hearnshaw, J. R., Glaister, D. H., Heffron, P. F. and Peck, A. W. Gustatory sweating following parotidectomy (T) 33*P*

Heffron, P. F., Glaister, D. H., Hearnshaw, J. R. and Peck, A. W. Gustatory sweating following parotidectomy (T) 33*P*

Hellon, R. F. and Lind, A. R. The influence of age on peripheral vasodilatation in a hot environment 262

Hill, I. M., Hirt, R. L., Tubbs, O. S. and Wells, B. G. The use of a Gibbon type 'heart-lung' machine in experimental cardiac surgery (T) 27*P*

Hirt, R. L., Hill, I. M., Tubbs, O. S. and Wells, B. G. The use of a Gibbon type 'heart-lung' machine in experimental cardiac surgery (T) 27*P*

Holman, Mollie E. Membrane potentials recorded with high-resistance microelectrodes; and the effects of changes in ionic environment on the electrical and mechanical activity of the smooth muscle of the taenia coli of the guinea-pig 464*P*

Holmes, R. L. and Coupland, R. E. The distribution of cholinesterase in the adrenal glands of the rat, cat and rabbit 97

Holmes, R. L. and Wolstencroft, J. H. Costocervical and thyrocervical contributions to the blood supply of the cat brain 12*P*

Holton, Pamela. The liberation of ATP from perfused rabbits' ears on antidromic stimulation of the sensory nerve 13*P*

Hope, D. B. Cystathionine in brain and liver from pyridoxine-deficient rats . 31*P*

Hsieh, A. C. L. The effects of hexamethonium bromide on temperature regulation in man 281

Hubbard, S. J. A study of rapid mechanical events in a mechano-receptor . 198

Hunt, J. N. and Murray, F. A. Changes in gastric function during pregnancy and lactation in women (T) 29*P*

Hussain, A. and Downman, C. B. B. Spinal tracts and supraspinal centres influencing visceromotor and allied reflexes in cats 489

Inman, D. R., Diamond, J. and Gray, J. A. B. The depression of the receptor potential in Pacinian corpuscles 117

Joyce, C. R. B. Measurement of reaction times with a conventional scaling unit 1*P*

Joyce, C. R. B. Differences between parts of packed human red cell column . 9*P*

Kärki, N. T. The effect of changes in ion concentration on ventricular fibrillation induced electrically 366

Kekwick, A., Chalmers, T. M. and Pawan, G. L. S. Observations on the fat-mobilizing activity of human urine during periods of food deprivation . . 9*P*

Kerslake, D. McK., Brebner, D. F. and Waddell, J. L. The relation between the coefficients for heat exchange by convection and by evaporation in man . 164

Kerslake, D. McK. and Waddell, J. L. The heat exchanges of wet skin . 156

Krnjević, K. and Miledi, R. Some effects produced by adrenaline upon neuromuscular propagation in rats 291

Lind, A. R. and Hellon, R. F. The influence of age on peripheral vasodilatation in a hot environment 262

Lindop, Patricia J. and Rotblat, J. Whole-body irradiation of animals by 15 MeV X-rays from the linear accelerator (T) 27*P*

Lindop, Patricia J. and Rotblat, J. A method of recording whole-body movement of mice in relation to ageing (T) 27*P*

Lindop, Patricia J. and Rotblat, H. The variation of radiation sensitivity with age 39*P*

PAGE

Lloyd, Sybil M. The responses of the vascular system in different phases of the reproductive cycle 37*P*

Lockett, Mary F. and Dimsdale, J. A preliminary investigation of the effect of isoprenaline on the breathing of chloralosed cats 42*P*

Lowy, J. and Abbott, B. C. Contraction in molluscan smooth muscle . . 385

Lowy, J. and Abbott, B. C. Mechanical properties of *Helix* and *Mytilus* muscle 398

McCance, R. A., Boylan, J. W. and Colbourn, Elizabeth P. Renal function in the foetal and new-born guinea-pig 323

McCance, R. A. and Widdowson, Elsie M. The response of the new-born puppy to water, salt and food 81

McCance, R. A. and Widdowson, Elsie M. The response of the new-born piglet to an excess of potassium 88

McDonald, D. A., Bergel, D. H. and Taylor, M. G. A method for measuring arterial impedance using a differential manometer 17*P*

Maclagan, Jennifer, Bigland, Brenda, Goetzee, Beryl and Zaimis, Eleanor. The effect of lowered muscle temperature on the action of neuromuscular blocking drugs 425

Marsden, J. L., Aumonier, F. J., Cowan, G. A. and Franklin, K. J. Variation in the response of human tissues to similar stimuli at different ages . . . 45*P*

Marshall, P. B., Cass, Rosemary and Riley, J. F. 5-Hydroxytryptamine and histamine in mast cells of the mouse and rat 510

Meade, F., Cotes, J. E. and Wise, M. E. Variations, in the vertical work done against gravity and in the energy expenditure of walking, between men and women and in relation to training 28*P*

Mendel, David. A method for the continuous determination of the pH of the blood 19*P*

Miledi, R. and Krnjević, K. Some effects produced by adrenaline upon neuromuscular propagation in rats 291

Munro, A. F. and Robinson, R. Normal levels for plasma adrenaline and noradrenaline compared with those in subjects with complete transverse lesions of the spinal cord 4*P*

Murray, F. A. and Hunt, J. N. Changes in gastric function during pregnancy and lactation in women (T) 29*P*

Muscholl, Erich and Vogt, Marthe. The action of reserpine on the peripheral sympathetic system 132

Nonweiler, T. The work production of man; studies on racing cyclists . . 8*P*

Nunn, Joan and Dicker, S. E. Antidiuresis in adult and old rats 332

O'Connor, W. J. The renal response of conscious dogs to the ingestion of 0·9% sodium chloride 6*P*

O'Connor, W. J. The renal response of conscious dogs to occlusion of the common carotid arteries 35*P*

Offerijns, F. G. J., Westerink, D. and Willebrands, A. F. The relation of potassium deficiency to muscular paralysis by insulin 377

Palmer, J. F., Fernandez de Molina, A. and Gray, J. A. B. Effects of acetylcholine on the activity of the lumbosacral cord of the cat 169

Paton, A., Cobbold, A. F., Galbraith, H. J. B. and Ginsburg, Jean. Circulatory, metabolic and respiratory effects of *iso*propylnoradrenaline in man . 40*P*

Pawan, G. L. S., Chalmers, T. M. and Kekwick, A. Observations on the fat-mobilizing activity of human urine during periods of food deprivation . . 9*P*

Peck, A. W., Glaister, D. H., Hearnshaw, J. R. and Heffron, P. F. Gustatory sweating following parotidectomy (T) 33*P*

PAGE

Peiss, Clarence N. Cardiovascular responses to electrical stimulation of the brain stem 500

Pickford, Mary and Abrahams, V. C. The effect of distension of the uterus on the composition of urine of conscious dogs 527

Pickford, Mary, Ali, M. N. and Cross, R. B. Electrolyte excretion in diuretic and non-diuretic dogs 177

Pugh, L. G. C. E. Muscular exercise on Mount Everest 233

Riley, J. F., Cass, Rosemary and Marshall, P. B. 5-Hydroxytryptamine and histamine in mast cells of the mouse and rat 510

Robinson, R. and Munro, A. F. Normal levels for plasma adrenaline and noradrenaline compared with those in subjects with complete transverse lesions of the spinal cord 4*P*

Rodnight, R. Carbon monoxide saturation and the recovery of 5-hydroxytryptamine from whole blood 10*P*

Rotblat, J. and Lindop, Patricia J. Whole-body irradiation of animals by 15 MeV X-rays from the linear accelerator (T) 27*P*

Rotblat, J. and Lindop, Patricia J. A method of recording whole-body movement of mice in relation to ageing (T) 27*P*

Rotblat, H. and Lindop, Patricia J. The variation of radiation sensitivity with age 39*P*

Scholes, N. W., Creese, R. and Hashish, S. E. E. Potassium in stimulated muscle 5*P*

Scott, Mary J. and Daly, M. de Burgh. The effects of stimulation of the carotid body chemoreceptors on heart rate in the dog 32*P*

Scowen, E. F., Archer, H. E. and Watts, R. W. E. Studies on primary hyperoxaluria 18*P*

Scowen, E. F., Donath, E. M. and Hadfield, J. The response of the male mouse breast to human mastotrophic substances 27*P*

Shephard, R. J. 'Breath-holding' measurement of carbon monoxide diffusing capacity. Comparison of a field test with steady-state and other methods of measurement 408

Smith, A. N., Black, J. W. and Fisher, E. W. Factors affecting histamine-stimulated gastric secretion in anaesthetized dogs 22

Smith, A. N., Black, J. W. and Fisher, E. W. The effects of 5-hydroxytryptamine on gastric secretion in anaesthetized dogs 27

Smith, A. N., Black, J. W. and Fisher, E. W. Factors affecting histamine-stimulated gastric secretion in anaesthetized dogs 42*P*

Smyth, D. H. and Taylor, C. B. Intestinal transfer of short-chain fatty acids *in vitro* 73

Styles, P. and Buller, A. J. A simple method of time coding for use with continuous film records 25*P*

Taylor, C. B. and Smyth, D. H. Intestinal transfer of short-chain fatty acids *in vitro* 73

Taylor, M. G. A simple electrical computer for Fourier analysis and synthesis . 23*P*

Taylor, M. G., Bergel, D. H. and McDonald, D. A. A method for measuring arterial impedance using a differential manometer 17*P*

Theobald, G. W. The separate release of oxytocin and ADH 30*P*

Thomas, J. G. An electrical method for recording eye movements 7*P*

Thompson, J. W. Studies on the responses of the isolated nictitating membrane of the cat 46

PAGE

Titchen, D. A. Reflex stimulation and inhibition of reticulum contractions in the ruminant stomach 1

Tubbs, O. S., Hill, I. M., Hirt, R. L. and Wells, B. G. The use of a Gibbon type 'heart-lung' machine in experimental cardiac surgery (T) 27*P*

Ullmann, Elisabeth. Osmotic diuresis during water diuresis 11*P*

Ullmann, Elisabeth and Currie, J. C. M. Polyuria during experimental modifications of breathing 43*P*

Vogt, Marthe and Muscholl, Erich. The action of reserpine on the peripheral sympathetic system 132

Waddell, J. L., Brebner, D. F. and Kerslake, D. McK. The relation between the coefficients for heat exchange by convection and by evaporation in man . 164

Waddell, J. L. and Kerslake, D. McK. The heat exchanges of wet skin . . 156

Wales, R. G. and White, I. G. The interaction of pH, tonicity and electrolyte concentration on the motility of dog spermatozoa 273

Watts, R. W. E., Archer, H. E. and Scowen, E. F. Studies on primary hyperoxaluria 18*P*

Wells, B. G., Hill, I. M., Hirt, R. L. and Tubbs, O. S. The use of a Gibbon type 'heart-lung' machine in experimental cardiac surgery (T) 27*P*

Westerink, D., Offerijns, F. G. J. and Willebrands, A. F. The relation of potassium deficiency to muscular paralysis by insulin 377

White, I. G. and Wales, R. G. The interaction of pH, tonicity and electrolyte concentration on the motility of dog spermatozoa 273

Whitteridge, D. The motor supply to the extra-ocular muscle spindles (T) . 33*P*

Widdas, W. F. and Bowyer, Freda. The action of inhibitors on the facilitated hexose transfer system in erythrocytes 219

Widdicombe, J. G. A method for recording changes in bronchial tone . . 20*P*

Widdicombe, J. G., Aumonier, F. J. and Crosfill, M. L. The relationship between lung compliance and histology in different species 20*P*

Widdowson, Elsie M. and McCance, R. A. The response of the new-born puppy to water, salt and food 81

Widdowson, Elsie M. and McCance, R. A. The response of the new-born piglet to an excess of potassium 88

Willebrands, A. F., Offerijns, F. G. J. and Westerink, D. The relation of potassium deficiency to muscular paralysis by insulin 377

Wise, M. E., Cotes, J. E. and Meade, F. Variations, in the vertical work done against gravity and in the energy expenditure of walking, between men and women and in relation to training 28*P*

Wolff, H. S. The accuracy of the integrating motor pneumotachograph (IMP) . 36*P*

Wolstencroft, J. H. and Holmes, R. L. Costocervical and thyrocervical contributions to the blood supply of the cat brain 12*P*

Zaimis, Eleanor and Bigland, Brenda. Factors influencing limb temperature during experiments on skeletal muscle 420

Zaimis, Eleanor, Bigland, Brenda, Goetzee, Beryl and Maclagan, Jennifer. The effect of lowered muscle temperature on the action of neuromuscular blocking drugs. 425

SUBJECT INDEX

Papers published in Proceedings by title only are indicated by (T)

Abomasum, reticulum contractions affected by acid in (ruminant), 6

Acetylcholine, ciliary movement affected by (frog), 520
  isolated nictitating membrane response to (cat), 50
  Renshaw cell response to electrophoretically-applied (cat), 438
  spinal cord activity affected by (cat), 169

Active state, plain muscle relaxation and (*Mytilus*), 393

Adrenal gland, cholinesterase distribution in (mammal), 97

Adrenal medulla, catechol amines distribution in homogenates of (bovine), 183
  chromaffin reaction in (rat), 193
  reserpine effect on catechol amines in (mammal), 146

Adrenaline, adrenal medulla homogenates distribution of (bovine), 184
  isolated nictitating membrane response to (cat), 50
  neuromuscular propagation affected by (rat), 291
  reserpine action on sympathetic ganglia (mammal), 136
  reticulum contractions affected by (ruminant), 14

Age, antidiuretic response affected by (rat), 332
  peripheral vasodilatation in a hot environment affected by (man), 262
  radiation sensitivity variation with (mouse), 39*P*
  response of tissues to similar stimuli affected by (man), 45*P*

Alveolar gas volume, pulmonary diffusing capacity measurement affected by (man), 411

Anaesthetic, nictitating membrane (isolated) sensitivity affected by previous (cat), 65

Anions, chloride output from skeletal muscle affected by other (frog), 351

Anticholinesterases, Renshaw cell response to electrophoretically-applied (cat), 447

Antidiuretic activity, —SH active compounds destruction in vasopressin of (pig), 111

Antidiuretic hormone, age effect on renal response to (rat), 332
  separate release of oxytocin and (man), 30*P*

Antidromic excitation, Pacinian corpuscle receptor potential affected by (cat), 123

Apparatus. Blood pH, continuous measurement, 19*P*

Arterial impedance, differential manometer in measurement of, 17*P*

Arterial wall, photo-electric method for determining elasto-viscous behaviour of, 22*P*

ATP, antidromic stimulation of sensory nerve liberating (rabbit), 13*P*

Blood-brain barrier, Renshaw cells and (cat), 446

Blood pH, method for continuous measurement of, 19*P*

Body irradiation, 15 MeV X-rays from linear accelerator for (T), 27*P*

Body movement, method of recording whole (mouse) (T), 27*P*

Body temperature, age effect on response to exercise in a hot environment of (man), 266
  wet skin temperature relation to (man), 160

Bone mast cells, histamine and 5-HT in (rat), 503

Brain, costocervical and thyrocervical contributions to blood supply of (cat), 12*P*

Brain stem, cardiovascular responses to stimulation of (cat, dog), 500

'Breath-holding', pulmonary diffusing capacity measurements by other methods compared with (man), 408

Bronchial tone, method for recording changes in, 20*P*

Calcium, plain muscle activity affected by changes in (guinea-pig), 480
  ventricular fibrillation affected by potassium and (rabbit), 368

Carbon monoxide, 'breath-holding' comparison with other methods of measurement of diffusing capacity of (man), 408

Cardiovascular response, brain stem stimulation invoking (cat, dog), 500

Carotid artery occlusion, renal response to (dog), 35*P*

Carotid body chemoreceptors, heart rate affected by stimulation of (dog), 32*P*
Cerebrospinal fluid pressure, head down tilting effect on (dog), 3*P*
Chloride, anions effect on output from skeletal muscle of tracer (frog), 351
  ventricular fibrillation affected by lack of (rabbit), 370
Cholinesterase, adrenal gland distribution of (mammal), 97
Chromaffin reaction, nature of, 193
Ciliary movement, tubocurarine and ACh action on (frog), 520
Climbing, efficiency relation to rate of (man), 249
Cycling, work production in racing (man), 8*P*
Cystathionine, pyridoxine deficiency effect on brain and liver (rat), 31*P*
Cysteine, antidiuretic activity of vasopressin inactivation by, 111

Decamethonium, Renshaw cell response to electrophoretically-applied (cat), 454
Decerebrate rigidity, visceromotor reflex paths and (cat), 492
Decerebration, reticulum contractions affected by (ruminant), 4
Defaecation, 5-HT and 5-HTP effect on (mouse), 14*P*
Dinitrofluorobenzene, inhibition of hexose transfer in erythrocytes by (man), 226
Dopamine, adrenal medulla homogenates distribution of (bovine), 188

Edrophonium, Renshaw cell response to electrophoretically-applied (cat), 448
Electrolyte concentration, spermatozoa motility affected by pH and (dog), 276
Electrolyte excretion, water diuresis effect on (dog), 177
End plate potentials, adrenaline action on (rat), 297
Erythrocytes, differences between parts of packed columns of (man), 9*P*
  inhibitors action on facilitated hexose transfer system in (man), 219
Everest, muscular exercise studies on expedition up (man), 233
Exercise, high altitude effect on oxygen consumption and ventilation associated with (man), 238
Extra-ocular muscle spindles, motor supply to (T), 33*P*
Eye movements, electrical method for recording (man), 7*P*

Fasting, fat-mobilizing activity of urine during (man), 9*P*
Fat-mobilization, fasting urine causing (man), 9*P*
Fatty acids, intestinal transfer of short-chain (rat), 73
Fish gut, temperature effect on movements of (trout), 35
Foetus, renal function in (guinea-pig), 323
Foot temperature, hexamethonium effect on (man), 284
Forearm blood flow, age effect on response to hot environment of (man), 262
Fourier analysis, simple electric computer for, 23*P*

Gastric function, pregnancy and lactation effect on (man) (T), 29*P*
Gastric secretion, factors affecting histamine-stimulated (dog), 22, 42*P*
  5-HT effect on (dog), 27
Glucose, fatty acids transfer in intestine affected by (rat), 76
Gustatory sweating, parotidectomy resulting in (T), 33*P*

Hand, retention of adaptation to warming and cooling of (T), 3*P*
Heart-lung machine, Gibbon type (T), 27*P*
Heart rate, carotid body chemoreceptors stimulation effect on (dog), 32*P*
Heat exchange coefficients, convection and evaporation (man), 164
Heat loss, evaporation and convection relation in (man), 166
Heat production, plain muscle contraction and (molluscs), 390
Hexamethonium, temperature regulation affected by (man), 281
Hexose transfer, inhibitors action in erythrocytes on (man), 219
High altitude, oxygen consumption and ventilation in exercise affected by (man), 238
Histamine, feeding effect on gastric secretion response to (dog), 22
  5-HT effect on gastric secretion response to (dog), 28
Hot environment, age effect on response of forearm blood flow to (man), 262
Hydroxytryptamine, carbon monoxide effect on recovery from blood of, 10*P*
  defaecation and gut propulsion affected by (mouse), 14*P*
  gastric secretion affected by (dog), 27
  isolated nictitating membrane response to (cat), 50

Hydroxytryptamine, mast cells containing (mouse, rat), 510
  vascular action on liver of (dog), 38*P*
Hyperoxaluria, primary (man), 18*P*

Insulin, potassium deficiency relation to muscular paralysis caused by (rat), 377
Integrating motor pneumotachograph, accuracy of (man), 36*P*
Intestine, short-chain fatty acids transfer in isolated (rat), 73
Isometric tension-length curve, plain muscle resting tension and (*Mytilus*), 399
Isoprenaline, breathing under chloralose affected by (cat), 42*P*
Isopropylnoradrenaline, circulatory, metabolic and respiratory effects of (man), 40*P*
  isolated nictitating membrane response to (cat), 53
Isotonic contraction, characteristics of plain muscle (molluscs), 386

Kidney, antidiuretic activity of vasopressin destruction by fractions of homogenate of (pig), 107
  excretory powers in foetus of (guinea-pig), 327
  uterine distension effect on excretory function of (dog), 527

Limb temperature, factors affecting (mammal), 420
Local tetanus intoxication, anaesthesia and (rat), 40*P*
Lung compliance, histology relation to (mammal), 20*P*.

Magnesium, ventricular fibrillation affected by (rabbit), 371
Mammotrophic substances, male breast response to (mouse), 27*P*
Mast cells, histamine and 5-HT in (mouse, rat), 510
Mechanoreceptors, properties of skin (frog), 305
  rapid mechanical events in (cat), 198
Medulla, cardiovascular responses to stimulation of (cat, dog), 500
  visceromotor reflexes influenced by centres in (cat), 495
Membrane potentials, magnitude in plain muscle of (guinea-pig), 464
Miniature end plate potentials, adrenaline action on (rat), 298
Mount Everest expedition, muscular exercise studies on (man), 233
Muscle, labelled substances collected from (T), 1*P*
Muscular paralysis, potassium deficiency relation to insulin causing (rat), 377

Neuromuscular block, temperature effect on (mammal), 425
Neuromuscular propagation, adrenaline action on (rat), 291
New-born, potassium excess intake effect on (pig), 88
  renal function in (guinea-pig), 323
  water, salt and food intake effect on balances in (dog), 81
Nicotine, isolated nictitating membrane response to (cat), 60
  Renshaw cell response to electrophoretically-applied (cat), 440
Nictitating membrane, responses of isolated (cat), 46
Noradrenaline, adrenal medulla homogenates distribution of (bovine), 184
  isolated nictitating membrane response to (cat), 50
  reserpine action on sympathetic ganglia (mammal), 134

Oesophagus, reticulum contractions affected by stimulation of (ruminant), 8
Omasum, reticulum contractions affected by stimulation of (ruminant), 8
Oxygen consumption, altitude effect on exercise maximum (man), 242
Oxytocin, separate release of ADH and (man), 30*P*

Pacinian corpuscle, rapid mechanical events in (cat), 198
  receptor potential depression in (cat), 117
Perfused hind-limb preparation (rat), 2*P*
Peristalsis, temperature effect on gut (trout), 38
pH, spermatozoa motility affected by NaCl and (dog), 276
Physical work, adaptation to exhausting (man), 35*P*
Plain muscle, contraction in (molluscs), 385
  ionic environment changes effect on activity of (guinea-pig), 464
  mechanical properties of (*Helix, Mytilus*), 398
Platelets, DNP and K effect on 5-HT uptake by (man), 39*P*
Polyphloretin phosphate, inhibition of hexose transfer in erythrocytes by (man), 226

Polyuria, modifications of breathing causing (man), 43*P*

Pons, cardiovascular responses to stimulation of (cat, dog), 500

Potassium, chloride output from skeletal muscle affected by (frog), 357
- new-born affected by excess intake of (pig), 88
- plain muscle activity affected by changes in (guinea-pig), 470
- ventricular fibrillation affected by calcium and (rabbit), 368

Potassium deficiency, muscular paralysis by insulin relation to (rat), 377

Presynaptic failure, adrenaline action on neuromuscular (rat), 293

Procaine, Renshaw cell response to electrophoretically-applied (cat), 450

Prostigmine, Renshaw cell response to electrophoretically-applied (cat), 447

Pulmonary diffusing capacity, 'breath-holding' comparison with other methods of measurement of carbon monoxide (man), 408

Pulmonary ventilation, altitude effect on exercise maximum (man), 242

Pylorus, reticulum contractions affected by stimulation of (ruminant), 13

Pyridoxine deficiency, brain and liver cystathionine affected by (rat), 31*P*

Reaction time, conventional scaling unit for measurement of (man), 1*P*

Receptor delay, skin mechanoreceptors showing (frog), 316

Receptor potential, depression in Pacinian corpuscle of (cat), 117

Renshaw cells, diffusional barriers around (cat), 446
- electrophoretically-applied stimulation of (cat), 435

Reproductive cycle, vascular system responses in different phases of (rat), 37*P*

Reserpine, peripheral sympathetic system affected by (mammal), 132

Respiration, polyuria during experimental modifications of (man), 43*P*

Reticulum, reflex stimulation and inhibition of contractions of (ruminant), 1

Saline, renal response to ingestion of (dog), 6*P*

Salt, new-born response to intake of (dog), 81

Sensory nerve, ATP liberation on antidromic stimulation of (rabbit), 13*P*

Skeletal muscle, anion interaction in (frog), 351
- factors affecting temperature of limb (mammal), 420
- potassium in 5*P*

Skeletal muscle contraction, chloride output affected by (frog), 359

Skin, heat exchanges of wet (man), 156

Skin mechanoreceptors, some properties of (frog), 305

Sodium, plain muscle activity affected by reduction of (guinea-pig), 479
- ventricular fibrillation affected by calcium and (rabbit), 369

Spatial summation, background intensity effect on visual (man), 337

Spermatozoa, pH, tonicity and electrolyte concentration effect on motility of (dog), 273

Spinal cord, acetylcholine action on activity of (cat), 169

Spinal cord transection, plasma catechol amines affected by (man), 4*P*
- visceromotor reflexes affected by (cat), 490

Splenic nerve, reserpine action on catechol amines in (mammal), 138

Strength-duration curve, skin mechanoreceptor (frog), 314

Sulphate, plain muscle activity affected by replacement of Cl by (guinea-pig), 476

Supraspinal centres, visceromotor reflexes influenced by (cat), 493

Suxamethonium, temperature effect on blocking action of (cat), 426

Sympathetic ganglia, reserpine effect on catechol amines in (mammal), 134

Sympathetic system, reserpine effect on peripheral (mammal), 132

Taenia coli, ionic environment changes effect on activity of (guinea-pig), 464

Temperature, fish gut movements affected by (trout), 35
- neuromuscular blocking agents' action affected by (mammal), 425
- plain muscle relaxation affected by (*Helix*), 404

Temperature regulation, hexamethonium action on (man), 281

Temporal summation, background intensity effect on visual (man), 337

Time coding, simple method for use with continuous film records of, 25*P*

Tubocurarine, ciliary movement affected by (frog), 520
- Renshaw cell response to electrophoretically-applied (cat), 449

Tubocurarine, temperature effect on blocking action of (cat, dog), 429

Urethane, inhibition of hexose transfer in erythrocytes by (man), 225
Urine creatinine, concentration in foetus of (guinea-pig), 326
Urine, uterine distension effect on volume and composition of (dog), 529
Uterine cervix, parturition effect on distensibility of (rat), 34*P*
Uterine distension, urine composition affected by (dog), 527

Vagotomy, gastric secretion response to histamine and 5-HT affected by (dog), 32
Vagus nerve stimulation, reticulum contractions affected by afferent (ruminant), 5
temperature effect on gut response to (trout), 41
Vasopressin, —SH active compounds destruction of antidiuretic activity of (pig), 107
Ventricle strip, thyroid state effect on contractility of isolated (rat), 29*P*
Ventricular fibrillation, ions effect on electrically-induced (rabbit), 366
Visceromotor reflexes, spinal tracts and supra-spinal centres influencing (cat), 489
Vision, background intensity effect on temporal and spatial summation in (man), 337

Walking, sex difference in energy expenditure of (man), 28*P*
Water diuresis, electrolyte excretion during (dog), 177
osmotic diuresis during (man), 11*P*
Water, new-born response to intake of (dog), 81